Patterns of Low Density Lipoprotein are Determinants in the Induction of Nitrooxidative Stress in Cardiovascular System

A dissertation presented to
the faculty of
the College of Arts and Sciences of Ohio University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy

Jiangzhou Hua

December 2015

© 2015 Jiangzhou Hua. All Rights Reserved.
This dissertation titled
Patterns of Low Density Lipoprotein are Determinants in the Induction of
Nitrooxidative Stress in Cardiovascular System

by

JIANGZHOU HUA

has been approved for
the Department of Chemistry and Biochemistry
and the College of Arts and Sciences by

Tadeusz Malinski
Distinguished Professor of Biochemistry

Robert Frank
Dean, College of Arts and Sciences
Abstract

HUA, JIANGZHOU, Ph.D., December 2015, Chemistry

Patterns of Low Density Lipoprotein are Determinants in the Induction of Nitrooxidative Stress in Cardiovascular System

Director of Dissertation: Tadeusz Malinski

Clinical research suggests that small and dense low density lipoprotein (LDL) may be correlated with increased cardiovascular risk. However, the effect of LDL with different patterns in the cardiovascular system, especially in endothelial cells, remains vague. In this study, we used nanosensors to measure real-time production of nitric oxide (NO) and peroxynitrite (ONOO⁻) and investigated their role in the regulation of cell adhesion and noxious effects on human umbilical vein endothelial cells (HUVECs).

Direct injection of LDL with different patterns (pattern A: 1.016-1.019 g/mL, pattern I: 1.024-1.029 g/mL, and pattern B: 1.034-1.053 g/mL) to HUVECs immediately generated ONOO⁻ and NO. The [NO] to [ONOO⁻] ratio was 2.7±0.4, 0.5±0.1 and 0.9±0.1 for pattern A, B and I, respectively. A ratio below 1.0 indicates a serious imbalance between cytoprotective NO and cytotoxic ONOO⁻. LDL (50% B and 50% I) stimulated endothelial cells to produce the highest concentration of ONOO⁻ (293±14 nmol/L) and lowest concentration of NO (166±10 nmol/L), whereas LDL composed of 60% A, 20% B and 20% I produced the lowest concentration of ONOO⁻ (77±8 nmol/L) and highest concentration of NO (436±28 nmol/L). All patterns of LDL upregulated the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Monocyte adhesion increased about 575%, 276%, and 194% (vs. control) in
the presence of 400 µg/mL of patterns B, I, or A LDL, respectively. Our results suggest that pattern B can stimulate endothelial cells to produce the highest level of ONOO⁻ and decrease NO to the lowest level, resulting in an imbalance between NO and ONOO⁻, followed by endothelial dysfunction.

Our results also suggest that endothelial cells of African Americans are more susceptible to LDL stimulation than endothelial cells of Caucasian Americans. Therefore, imbalance between NO and ONOO⁻ in the presence of LDL, followed by endothelial dysfunction, and upregulation of ICAM-1 and VCAM-1 expression are more significant in endothelium of African Americans than in endothelium of Caucasian Americans.

LDL patterns and their relative distribution are determinants of endothelial function or dysfunction, the concentration of bioavailable NO, nitrooxidative stress and the expression of adhesion molecules. Therefore, an unfavorable distribution of pattern A, B and I in LDL may be considered as an important risk factor in atherosclerosis.
Dedication

I dedicate this work to my beloved parents, grandma and my cat (miaomiao) as a token of affection and gratitude.
Acknowledgments

First, I would like to express my sincerest gratitude to my advisor Dr. Tadeusz Malinski. He gave me the great opportunity to join his research group. I will always be proud of being a member in his laboratory. He has taught me the necessary skills to be a better scientist. His innovations and ideas are amazing and I am grateful for the guidance he has given me and will always remember that. I could not have completed this project without his encouragement and support. I am extremely thankful for all of that.

I would like to thank my committee members, Dr. Hao Chen, Dr. Michael Held and Dr. Tiao J. Chang, for all their valuable time and work spent on the committee meetings, and the final dissertation review, and for their helpful advice to my research project. I would like to thank all the past and current members in my research group: Dr. Adam Jacoby, Dr. Han Wang, Dr. Salah Awad, Dr. Lulin Jiang, Yuanyuan Tang, Alamzeb Khan, Farina Mahmud, Hazem Dawould, Mike Wagner, Taghi Sahraeian and Osama Muhammad, with whom it is enjoyable to work with and for their support and encouragement all the time.

I would also like to give thanks to the faculty and staff of the Department of Chemistry and Biochemistry. Special thanks to Paula Hale, Marlene Jenkins, Rollie Merriman, Aaron Dillon, Leslie Scripp and Carolyn Khurshid. Special thanks to Paul Schmittauer and Bascom French.

Finally, deepest gratitude to my parents, relatives and friends in China, for their unrelenting love, sacrifice, support and tolerance. The last, to my sweet cat, miaomiao, wish you rest in peace.
Table of Contents

Abstract ............................................................................................................................... 3
Dedication ........................................................................................................................... 5
Acknowledgments ............................................................................................................... 6
List of Tables .................................................................................................................... 10
List of Figures ................................................................................................................... 11
List of Abbreviations ........................................................................................................ 17
1 Introduction ............................................................................................................... 19
   1.1 Cardiovascular diseases .................................................................................... 19
   1.2 Nitric oxide ....................................................................................................... 21
      1.2.1 Nitric oxide measurement ........................................................................ 21
      1.2.2 Nitric oxide biological functions and synthesis ....................................... 23
      1.2.3 Nitric oxide synthase ............................................................................. 24
   1.3 Peroxynitrite ...................................................................................................... 27
   1.4 Low density lipoprotein .................................................................................... 29
      1.4.1 Lipoproteins .............................................................................................. 29
      1.4.2 Characterization of LDL ........................................................................... 31
      1.4.3 LDL subtype patterns ............................................................................. 32
      1.4.4 LDL physiology ..................................................................................... 33
      1.4.5 Advances in clinical measurement of LDL ............................................. 36
   1.5 Endothelial dysfunction .................................................................................... 38
   1.6 Research significance and goals ....................................................................... 39
2 Materials and methods .............................................................................................. 43
   2.1 Cell cultures ...................................................................................................... 43
   2.2 Electrochemical measurements for NO and ONOO⁻ ...................................... 43
      2.2.1 Principles of NO detection ..................................................................... 43
      2.2.2 Preparation of NO nanosensor ............................................................... 44
      2.2.3 Calibration of NO nanosensor ............................................................... 46
      2.2.4 Principles of ONOO⁻ detection .............................................................. 48
2.2.5 Preparation of ONOO⁻ nanosensor ............................................................... 49
2.2.6 Calibration of ONOO⁻ nanosensor ............................................................. 51
2.3 Isolation, oxidation and analysis of LDL ......................................................... 53
  2.3.1 LDL subpatterns isolation and oxidation .................................................. 53
  2.3.2 LDL analysis ............................................................................................. 54
2.4 Determination of n-LDL/ox-LDL induced NO and ONOO⁻ release from
  endothelial cells ................................................................................................. 55
2.5 Cell ELISA ...................................................................................................... 57
2.6 Monocyte adhesion ......................................................................................... 58
2.7 Statistical analysis .......................................................................................... 61
3 Results ............................................................................................................... 62
  3.1 Isolation, oxidation and analysis of LDL ......................................................... 62
  3.2 Determination of n-LDL/ox-LDL induced NO and ONOO⁻ release from
   endothelial cells ................................................................................................. 63
    3.2.1 n-LDL induced NO and ONOO⁻ release in endothelial cells ................ 63
    3.2.2 Ox-LDL induced NO and ONOO⁻ release in endothelial cells .......... 76
    3.2.3 n-LDL/ox-LDL induced NO and ONOO⁻ release in endothelial cells of
       African Americans (AA) and Caucasian Americans (CA) ......................... 78
  3.3 Effects of LDL on ICAM-1 and VCAM-1 expression in HUVECs ............... 86
    3.3.1 Effects of n-LDL on ICAM-1 and VCAM-1 expression in HUVECs .... 86
    3.3.2 Effects of ox-LDL on ICAM-1 and VCAM-1 expression in HUVECs .... 87
    3.3.3 Effects of n-LDL on ICAM-1 and VCAM-1 expression in HUVECs from
       AA and CA ................................................................................................. 89
  3.4 LDL induced cell adhesion in endothelial cells .............................................. 93
    3.4.1 n-LDL induced cell adhesion in endothelial cells .................................. 93
    3.4.2 Ox-LDL induced cell adhesion in endothelial cells .............................. 95
    3.4.3 n-LDL and ox-LDL induced cell adhesion in HUVECS of African
       Americans and Caucasian Americans ......................................................... 96
4 Discussion ....................................................................................................... 99
  4.1 Separation and oxidation of LDL subpatterns ............................................. 99
  4.2 n-LDL/ox-LDL induced an imbalance of NO and ONOO⁻ in endothelial cells
       101
    4.2.1 n-LDL stimulated NO and ONOO⁻ release in endothelial cells .......... 101
4.2.2 Ox-LDL induced NO and ONOO⁻ release from endothelial cells .......... 107
4.2.3 n-LDL/ox-LDL induced NO and ONOO⁻ release in endothelial cells from African Americans and Caucasian Americans ........................................................ 109

4.3 Effects of LDL on ICAM-1 and VCAM-1 expression in HUVECs .......... 111
4.3.1 Effects of n-LDL on ICAM-1 and VCAM-1 expression in HUVECs ...... 111
4.3.2 Effects of ox-LDL on ICAM-1 and VCAM-1 expression in HUVECs ..... 112
4.3.3 Effects of n-LDL on ICAM-1 and VCAM-1 expression in HUVECs of African Americans and Caucasian Americans ........................................................ 113

4.4 LDL induced cell adhesion in endothelial cells .................................... 114
4.4.1 n-LDL induced cell adhesion in endothelial cells ............................. 114
4.4.2 Ox-LDL induced cell adhesion in endothelial cells ........................... 115
4.4.3 n-LDL/ox-LDL induced cell adhesion in HUVECs of African Americans and Caucasian Americans ................................................................. 116

5 Conclusion ......................................................................................... 117

6 Future work ....................................................................................... 120

References .............................................................................................. 121
List of Tables

Table 1.1 Classification and biological function of NOS.\textsuperscript{28, 30, 31} ........................................... 26

Table 1.2 Composition and Density of Human Lipoproteins.\textsuperscript{69} ............................................. 31

Table 1.3 Classification and risk interpretation of different LDL cholesterol level.\textsuperscript{105} .... 38
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Factors Contributing to Cardiometabolic Risk. (Modified from&lt;sup&gt;8&lt;/sup&gt;)</td>
<td>20</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Oxidative stress-induced changes in physiological surroundings. (Modified from&lt;sup&gt;26&lt;/sup&gt;)</td>
<td>24</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>NOS dimer structure with cofactors and substrate binding sites. (Modified from&lt;sup&gt;37&lt;/sup&gt;)</td>
<td>27</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Isomerization and cleavage of peroxynitrous acid (ONOOH) at low and high concentrations in physiological surroundings. (Modified from&lt;sup&gt;26&lt;/sup&gt;)</td>
<td>28</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>The structure and components of lipoprotein. (Modified from&lt;sup&gt;68&lt;/sup&gt;)</td>
<td>30</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>The schematic diagram of NO electrochemical nanosensors. (Adapted from&lt;sup&gt;128&lt;/sup&gt;)</td>
<td>45</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Multiple-scan cyclic voltammograms for electrochemical deposition of a polymeric Ni-TMHPP on the tip of the carbon fibers. Peak I&lt;sub&gt;O&lt;/sub&gt; indicates oxidation of Ni (II) to Ni (III) and peak I&lt;sub&gt;R&lt;/sub&gt; indicates reduction of Ni (III) to Ni (II). Rising current (I&lt;sub&gt;a&lt;/sub&gt;) indicates catalytic oxidation of H&lt;sub&gt;2&lt;/sub&gt;O.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Experimental setup for synthesis of NO standard solution. (Adapted from&lt;sup&gt;130&lt;/sup&gt;)</td>
<td>47</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>The standard calibration curve of NO nanosensor (R&lt;sup&gt;2&lt;/sup&gt;=0.9917, n=3)</td>
<td>48</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>The schematic diagram of ONOO&lt;sup&gt;-&lt;/sup&gt; electrochemical nanosensors&lt;sup&gt;131&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Multiple-scan cyclic voltammograms for electrochemical deposition on the tip of carbon fibers. Peak I&lt;sub&gt;O&lt;/sub&gt; indicates oxidation of Mn (III) to Mn (IV) and peak I&lt;sub&gt;R&lt;/sub&gt; indicates reduction of Mn (IV) to Mn (III).</td>
<td>50</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Experimental setup for synthesis of ONOO&lt;sup&gt;-&lt;/sup&gt; standard (adapted from&lt;sup&gt;134&lt;/sup&gt;)</td>
<td>52</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>The standard calibration curve of ONOO&lt;sup&gt;-&lt;/sup&gt; nanosensor (R&lt;sup&gt;2&lt;/sup&gt;=0.9977, n=3).</td>
<td>52</td>
</tr>
<tr>
<td>Figure 2.9</td>
<td>Schematic diagram for separation of LDL subfractions from plasma.</td>
<td>54</td>
</tr>
</tbody>
</table>
Figure 2.10 Schematic diagram for monocyte adhesion assay. ........................................ 61

Figure 3.1 Density profile generated by iodixanol gradient solution after 4 hours ultracentrifugation (n=4) ................................................................................................... 62

Figure 3.2 Cholesterol standard curve (R^2=0.9944, n=3). ........................................ 63

Figure 3.3 Amperograms of NO release from endothelial cells stimulated with direct LDL injection (LDL of Pattern A, B and I, 1000 µg/mL). ........................................ 64

Figure 3.4 Amperograms of ONOO^- release from endothelial cells stimulated with direct LDL injection (LDL of Pattern A, B and I, 1000 µg/mL). ........................................ 65

Figure 3.5 Maximal [NO] and [ONOO^-] released from endothelial cells after LDL injection (LDL of Pattern A, I and B, 1000 µg/mL). NO (solid bars) and ONOO^- (open bars). Data are expressed as mean±SD. *P<0.01 vs control (n=4) ................................ 66

Figure 3.6 A ratio of maximal [NO] to [ONOO^-] produced by endothelial cells after LDL injection (LDL of Pattern A, B and I, 1000 µg/mL). Data are expressed as mean±SD. *P<0.01 vs control (n=4) .......................................................... 67

Figure 3.7 Dose-dependent NO release from the surface of endothelial cells after direct injection of LDL with different patterns (A, B and I) and concentrations (from 50 µg/mL to 1000 µg/mL). Data are expressed as mean±SD ................................................................. 68

Figure 3.8 Dose-dependent ONOO^- release from the surface of endothelial cells after direct injection of LDL with different patterns (A, B and I) and concentrations (from 50 µg/mL to 1000 µg/mL). Data are expressed as mean±SD.......................... 69

Figure 3.9 The ratio of [NO] to [ONOO^-] measured in endothelial cells after direct injection of LDL with different patterns (A, B and I) and concentrations (from 50 µg/mL to 1000 µg/mL). Data are expressed as mean±SD ......................................................... 70

Figure 3.10 [NO] and [ONOO^-] release from endothelial cells stimulated by LDL injection with different mixture combinations (800 µg/mL). Close bar indicates NO and open bar indicates ONOO^- Data are expressed as mean±SD. .................................................. 71

Figure 3.11 Ratio of [NO] to [ONOO^-]. Data are expressed as mean±SD ................ 72
Figure 3.12 NO release from endothelial cells stimulated by LDL injection (Pattern A, I and B, 800 µg/mL) after incubation with different treatments. Endothelial cells were pre-treated with control (EBM), PEG-SOD (400 U/mL), L-arginine (300 µM), sepiapterin (200 µM), L-NAME (100 µM) and VAS2870 (10 µM) at 37 ºC for 30 minutes. Data are expressed as mean±SD. 73

Figure 3.13 ONOO⁻ release from endothelial cells stimulated by LDL injection (Pattern A, I and B, 800 µg/mL) after incubation with different treatments. Endothelial cells were pre-treated with control (EBM), PEG-SOD (400 U/mL), L-arginine (300 µM), sepiapterin (200 µM), L-NAME (100 µM) and VAS2870 (10 µM) at 37 ºC for 30 minutes. Data are expressed as mean±SD. 74

Figure 3.14 Ratio of [NO] to [ONOO⁻]. Data are expressed as mean±SD. 75

Figure 3.15 NO production induced by ox-LDL/n-LDL (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs n-LDL (n=4). 76

Figure 3.16 ONOO⁻ production induced by ox-LDL/n-LDL (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs n-LDL (n=4). 77

Figure 3.17 Ratio of [NO] to [ONOO⁻]. Data are expressed as mean±SD. 77

Figure 3.18 Differences in peak NO release from HUVECs of African Americans (solid bars) and Caucasian Americans (open bars). Stimulation with n-LDL of pattern A, I and B (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4). 78

Figure 3.19 Differences in peak ONOO⁻ release from HUVECs of African Americans (solid bars) and Caucasian Americans (open bars). Stimulation with n-LDL of pattern A, I and B (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4). 79

Figure 3.20 Ratio of [NO] to [ONOO⁻]. HUVECs of African Americans (solid bars) and Caucasian Americans (open bars). Data are expressed as mean±SD. 80

Figure 3.21 Maximal NO released from endothelial cells of African Americans (solid bars) and Caucasian Americans (open bars). Stimulation with ox-LDL of pattern A, B
and I (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4). .......................................................... 81

Figure 3.22 Maximal ONOO- released from endothelial cells of African Americans (solid bars) and Caucasian Americans (open bars). Stimulation with ox-LDL of pattern A, B and I (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4). .......................................................... 82

Figure 3.23 Ratio of [NO] to [ONOO-]. HUVECs of African Americans (solid bars) and Caucasian Americans (open bars). Data are expressed as mean±SD. ............................. 83

Figure 3.24 NO release from HUVECs of African Americans (solid bars) and Caucasian Americans (open bars) after stimulation with n-LDL of different mixture combinations (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4).......................................................... 84

Figure 3.25 ONOO- release from HUVECs of African Americans (solid bars) and Caucasian Americans (open bars) after stimulation with n-LDL of different mixture combinations (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4). .......................................................... 85

Figure 3.26 Ratio of [NO] to [ONOO-]. African Americans (solid bars) and Caucasian Americans (open bars). Data are expressed as mean±SD................................................ 86

Figure 3.27 Effect of n-LDL incubation with different patterns on the expression of ICAM-1 (solid bars) and VCAM-1 (open bars). Data are expressed as mean±SD. OD_{450} indicates optical density at 450 nm wavelength. *P<0.01 vs control (n=6). ................. 87

Figure 3.28 Effect of different subpatterns of ox-LDL/n-LDL on the expression of ICAM-1. Data are expressed as mean±SD. OD_{450} indicates optical density at 450 nm wavelength. *P<0.01 vs control (n=6). ......................................................... 88

Figure 3.29 Effect of different subpatterns of ox-LDL/n-LDL on the expression of VCAM-1. Data are expressed as mean±SD. OD_{450} indicates optical density at 450 nm wavelength. *P<0.01 vs control (n=6)......................................................... 89

Figure 3.30 ICAM-1 expression stimulated by n-LDL of pattern A, I and B (400 µg/mL). OD_{450} indicates optical density at 450 nm wavelength. African Americans (solid bars)
and Caucasian Americans (CA). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6). .............................................................................................................. 90

Figure 3.31 VCAM-1 expression induced by n-LDL of pattern A, I and B (400 µg/mL). OD$_{450}$ indicates optical density at 450 nm wavelength. African Americans (solid bars) and Caucasian Americans (CA). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6). .............................................................................................................. 91

Figure 3.32 ICAM-1 expression induced by ox-LDL of pattern A, I and B (400 µg/mL). OD$_{450}$ indicates optical density at 450 nm wavelength. African Americans (solid bars) and Caucasian Americans (CA). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6). .............................................................................................................. 92

Figure 3.33 VCAM-1 expression induced by ox-LDL of pattern A, I and B (400 µg/mL). OD$_{450}$ indicates optical density at 450 nm wavelength. African Americans (solid bars) and Caucasian Americans (CA). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6). .............................................................................................................. 93

Figure 3.34 Monocyte adhesion induced by pattern A, I and B LDL (400 µg/mL) measured at different incubation times. ............................................................................ 94

Figure 3.35 Dose-dependent monocyte adhesion induced by incubation with n-LDL of pattern A, I and B (50, 100, 200, 400 µg/mL). Data are expressed as mean±SD. MFI indicates mean fluorescence intensity................................................................. 95

Figure 3.36 Monocyte adhesion induced by n-LDL/ox-LDL of pattern A, I and B (400 µg/mL). Data are expressed as mean±SD. MFI indicates mean fluorescence intensity. *P<0.01 vs n-LDL (n=6). .............................................................................................................. 96

Figure 3.37 Race-specific monocyte adhesion stimulated by different n-LDL subpatterns (400 µg/mL). MFI indicates mean fluorescence intensity. African Americans (solid bars) and Caucasian Americans (open bars). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6). .............................................................................................................. 97

Figure 3.38 Race-specific monocyte adhesion stimulated by different ox-LDL subpatterns (400 µg/mL). MFI indicates mean fluorescence intensity. African Americans (solid bars)
and Caucasian Americans (open bars). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6).

Figure 5.1 Effect of different LDL subpatterns on endothelium.

Figure 5.2 Race-specific difference in response to stimulation of different LDL subpatterns.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag/AgCl</td>
<td>Silver/silver chloride electrode</td>
</tr>
<tr>
<td>BCECF-AM</td>
<td>2’, 7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyl-</td>
</tr>
<tr>
<td></td>
<td>fluorescein acetoxy methyl ester</td>
</tr>
<tr>
<td>BH₄</td>
<td>5, 6, 7, 8-tetrahydrobiopterin</td>
</tr>
<tr>
<td>CaI</td>
<td>Calcium Ionophore</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>cNOS</td>
<td>Constitutive nitric oxide synthase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial basal medium</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>L-Arg</td>
<td>L-Arginine</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-N⁶-arginine methyl ester</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide (Oxidized form)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (Reduced form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>n-LDL</td>
<td>Native-Low density lipoprotein</td>
</tr>
<tr>
<td>ox-LDL</td>
<td>Oxidized-Low density lipoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG-SOD</td>
<td>Polyethylene glycol covalently linked to superoxide dismutase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TMB</td>
<td>4,4'-Bi-2,6-xylidine;4,4'-Diamino-3,3',5,5'-tetramethylbiphenyl</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Cardiovascular diseases

Cardiovascular disease (CVD) is the primary cause of death in the United States and many other developed countries worldwide. The percentage of premature deaths from CVD range from 4% in high-income countries to 42% in low-income countries. As of 2008, it is estimated that there were 17.3 million people had died from CVDs, with over 80% of which taking place in developing countries. By 2030, it is expected that the number of people who will die from CVD (mainly from heart disease and stroke) will increase to 23.3 million.

CVD is a class of diseases caused by disorders of the heart and blood vessels, which include heart attacks, stroke, hypertension and heart failure. In 1949, Jerry Morris first studied cardiovascular health by using occupational health data and his results were published in 1958. In the past several decades, considerable efforts have been made to identify, classify and modify risk factors for CVDs, which include older age, gender, air pollution, hypertension, dyslipidemia, obesity, type 2 diabetes, cigarette smoking and physical inactivity (Figure 1). Despite the diversification of the causes of cardiovascular disease, atherosclerosis is the most common. The atherosclerotic process occurs when an artery wall thickens due to the invasion and accumulation of macrophages as well as other specialized white blood cells (WBCs).

In addition, when people get old, a number of physiological changes inside the human body will affect cardiovascular function, leading to increased risk of CVD, even in individuals who are healthy or asymptomatic. It is estimated that 82% of the deaths
from CVD occur in people who are 65 and older. Meanwhile, the risk of stroke doubles every decade after age 55.\(^7\)

![Figure 1.1 Factors Contributing to Cardiometabolic Risk. (Modified from\(^8\))](image)

Consequently, more and more research interests have been focused on preventing CVD by modifying risk factors. According to the World Health Organization (WHO), there are many ways to reduce most of the CVD risk factors, including eating a healthy diet, doing physical exercise, and avoiding smoking tobacco and excessive alcohol intake. If those main risk factors were successfully eliminated, 80% of all heart disease and stroke would be prevented.\(^9\) People who are at high risk of CVD need early diagnosis and management using counselling and medicines, such as taking antioxidant supplementation (vitamin E, vitamin C, etc.). In addition, statins are effective in preventing further CVD for those who have cardiovascular events.\(^10\)
However, traditional risk factors do not account for the entirety of risk and there are many people who are considered as “low risk” according to the traditional definition, yet have suffered from CVD events. Therefore, hundreds of scientific studies have been carried out weekly for the purpose of elucidating the unclear fields associated with the pathogeny, precaution and remedy of CVDs.

1.2 Nitric oxide

1.2.1 Nitric oxide measurement

Nitric oxide (NO) is a free radical due to the existence of single unpaired electrons in its molecule, which makes it not only quite reactive, but also easily oxidized to nitrite and/or nitrate by oxygen, not to mention it has a short half-life ($t_{1/2}=3\text{-}6s$) in biological systems as well as its rapid diffusion.\(^{11}\) All of those unique chemical and physical properties make NO difficult to measure its concentration instantly, especially for \emph{in vivo} experiments. NO concentration can be measured by its reaction with ozone ($O_3$).\(^{12}\) A sample solution containing NO is mixed with an excess amount of $O_3$. Then NO reacts with ozone to produce oxygen ($O_2$) and nitrogen dioxide ($NO_2$) in excited state ($NO_2^*$). $NO_2^*$ is not stable and easily decayed to its ground state, accompanied by the emission of photon (600-875 nm), which can be measured with a photo sensor. The chemiluminescence intensity is proportional to the concentration of NO in the sample (eq.1.1).\(^{13}\)

$$NO + O_3 \rightarrow NO_2 + O_2 + hv$$ \hspace{1cm} (1.1)

Another popular way to determine NO concentration is the Griess assay (diazotization), which has advantages of low cost, easy operation and simple data
analysis. The Griess assay actually measures nitrite instead of measuring NO directly (eq.1.2-1.4).\textsuperscript{14}

\begin{align*}
2\text{NO} + \text{O}_2 & \rightarrow 2\text{NO}_2 \\
\text{NO} + \text{NO}_2 & \rightarrow \text{N}_2\text{O}_3 \\
\text{N}_2\text{O}_3 + \text{H}_2\text{O} & \rightarrow 2\text{NO}_2^- + 2\text{H}^+ 
\end{align*}

The original reaction involved nitrite, sulfanilic acid and α-naphthylamine under acidic conditions to generate an azo dye. Then the concentration of azo dye could be determined by measuring the absorbance at 540 nm and used as an indirect indicator of nitrite (and NO) concentration in the sample.\textsuperscript{14} However, the Griess assay is not suitable for real-time measurement due to the multi-step chemical reactions and the indirect detection. Furthermore, the presence of nitrate (NO\textsubscript{3}^-) as the possible decomposition byproduct from the reaction of NO and oxygen may cause low NO values.\textsuperscript{15}

Comparing to Griess assay and chemiluminescence assay, electrochemistry seems to be the superior way of measuring NO because of the capability of direct real-time and \textit{in situ} NO analysis. The electrochemical detection of NO is carried out via the one-step electron transfer of NO oxidized to NO\textsuperscript{+} (eq.1.5 and 1.6).\textsuperscript{11, 16}

\begin{align*}
\text{NO} - \text{e} & \rightarrow \text{NO}^+ \\
\text{NO}^+ + \text{OH}^- & \rightarrow \text{HNO}_2 
\end{align*}

A nanosensor designed by Malinski used conductive polymeric porphyrin to oxidize NO electrochemically at about 0.6 V vs. SSCE. Polymeric porphyrin was covered with Nafion to prevent diffusion of NO\textsubscript{2}^- to the surface of electrode and be oxidized to
NO₃⁻. Resultant current from the oxidation of NO measured by the sensor is converted to concentration of NO by utilization of a calibration curve.¹⁷,¹⁸

1.2.2 Nitric oxide biological functions and synthesis

NO generated from automobiles, industries and power stations is considered as an air pollutant which causes acid rain, smog and the vanishing of the ozone layer. NO reacts with O₂ and O₃ to produce NO₂, which is an environmental pollutant. Although NO is one of the toxic environmental pollutants, it is also a known bio-product in almost all types of organisms, including but not limited to bacteria, fungi, plants and animal cells.¹⁹ NO is a key cellular signaling messenger taking part in many physiological and pathological processes.²⁰ In 1978, Furchgott first discovered a substance produced and released from the endothelium to promote smooth muscle relaxation, which he named as the endothelium-derived relaxing factor (EDRF).²¹ Further studies of EDRF into its properties and mechanism of action revealed that it was in fact NO, a major factor in many aspects of cardiovascular physiology, including regulation of vasomotor tone,²² cell adhesion to the endothelium,²³ proliferation of vascular smooth muscle cell²⁴ and inhibition of platelet aggregation.²⁵

Although NO is beneficial for preventing cardiovascular damage, as a free radical, it can react with superoxide to produce ONOO⁻, which is highly oxidative and can cause damages to deoxyribonucleic acid (DNA) and proteins as well as lipid.²⁶
Figure 1.2 Oxidative stress-induced changes in physiological surroundings. (Modified from\(^\text{26}\))

DNA=deoxyribonucleic acid; eNOS= endothelial nitric oxide synthase; NO=nitric oxide; \(O_2^-=\)superoxide; \(\text{ONOO}^-\)=peroxynitrite.

**1.2.3 Nitric oxide synthase**

Nitric oxide is biosynthesized from L-arginine, oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) by nitric oxide synthase (NOS). Biosynthesis of NO catalyzed by NOS involves a two-step oxidation of L-arginine (L-Arg). First, L-Arg is oxidized to Nω-hydroxy-L-arginine (NOHLA) as an intermediate product, then NOHLA is further oxidized to generate L-citrulline and NO as final products.\(^\text{27}\) Nitric oxide synthases (NOSs) are a family of enzymes that catalyze the oxidation reaction of L-Arginine to generate NO (eq.1.7).\(^\text{26}\)

\[
2 \text{L-arginine} + 3 \text{NADPH} + 2H^+ + 4 O_2 \overset{\text{NOS}}{\leftrightarrow} 2 \text{citrulline} + 2 \text{NO} + 3 \text{NADP}^+ \quad (1.7)
\]
Different NOS isoforms are encoded by distinct genes. There are three well-studied isoforms in mammals which differ in structure and function. Two isoforms are called constitutive NOSs (cNOS) which are Ca\(^{2+}\)-dependent enzymes, and the third one is inducible NOS (iNOS) which is independent of Ca\(^{2+}\) concentration. Recently, NOS activity has been detected in several bacterial species, which attributes bacterial NOS (bNOS) as the fourth member to the NOS family.

Neuronal NOS (nNOS/NOS1) produces NO in nervous systems, plays an important role in cell signaling and is in association with plasma membranes. Distinct from constitutive NOS enzymes (nNOS and eNOS), iNOS (NOS2) is considered as calcium-insensitive, because it has tight non-covalent interaction with Ca\(^{2+}\) and calmodulin (CaM). iNOS is expressed at high level and produces large amount of NO after stimulation by several cytokines during inflammation, such as Interleukin-1 (IL-1), Tumor necrosis factor alpha (TNF-α) and Interferon gamma (IFN-γ). The high-output iNOS expression usually happens in an oxidative environment, and thus high concentration of NO release may react with superoxide (O\(_2^-\)) to produce peroxynitrite (ONOO\(^{-}\)), which is cytotoxic. These properties may reveal the roles that iNOS plays in host immune responses, such as its capability of participating in anti-microbial and anti-tumor activities.

Endothelial NOS (eNOS/NOS3) produces NO at a basal level in blood vessels of all types and is responsible for regulating vascular function by inhibiting smooth muscle contraction and platelet aggregation. It is a constitutive Ca\(^{2+}\) dependent NOS which has consensus structures with nNOS, including NADPH, flavin mononucleotide (FMN),
flavin adenine dinucleotide (FAD), and calmodulin binding sites and a protein kinase A (PKA) phosphorylation site.\textsuperscript{34} Similar to nNOS, eNOS is in association with plasma membranes as well as the intracellular membranes of Golgi bodies. Bacterial NOS (bNOS) plays an important role in superoxide dismutase (SodA) transcription. Bacterial lacking bNOS cannot upregulate SOD A expression, subjecting them to high levels of oxidative stress. Therefore, bNOS may be responsible for protecting the bacteria against oxidative stress, diverse antibiotics, conventional antimicrobials and host immune response.\textsuperscript{35, 36} The different forms of NOSs have been summarized as follows:\textsuperscript{34}

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene (s)</th>
<th>Location</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuronal NOS</td>
<td>NOS1</td>
<td>nervous tissue</td>
<td>cell communication</td>
</tr>
<tr>
<td>(nNOS or NOS1)</td>
<td>(Chromosome 12)</td>
<td>skeletal muscle type II</td>
<td></td>
</tr>
<tr>
<td>Inducible NOS</td>
<td>NOS2</td>
<td>immune system</td>
<td>immune defense against pathogens</td>
</tr>
<tr>
<td>(iNOS or NOS2)</td>
<td>(Chromosome 17)</td>
<td>cardiovascular system</td>
<td></td>
</tr>
<tr>
<td>Endothelial NOS</td>
<td>NOS3</td>
<td>endothelium</td>
<td>vasodilation</td>
</tr>
<tr>
<td>(eNOS or NOS3)</td>
<td>(Chromosome 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial NOS</td>
<td>multiple</td>
<td>various Gram-positive</td>
<td>defense against oxidative stress, antibiotics, immune attack</td>
</tr>
<tr>
<td>(bNOS)</td>
<td></td>
<td>bacteria</td>
<td></td>
</tr>
</tbody>
</table>

The normal NOS in its functional status is a dimer consisting of two identical monomers. Each monomer contains two major domains: an N-terminal oxygenase domain including an activation site for L-Arg binding and three cofactors of heme, zinc
ion and tetrahydrobiopterin (BH$_4$); and a C-terminal reductase domain with multi-binding sites of NADPH, FAD and FMN (Figure 3). There is a calmodulin (CaM)-binding region between the oxygenase and reductase domains, which shows distinct dependence on Ca$^{2+}$ among nNOS, eNOS and iNOS. Constitutive NOS (nNOS and eNOS) can be activated by increased intracellular concentration of Ca$^{2+}$, followed by the binding of Ca$^{2+}$/CaM. However, CaM bound within iNOS is irreversible, which makes iNOS independent of Ca$^{2+}$.

Figure 1.3 NOS dimer structure with cofactors and substrate binding sites. (Modified from$^{37}$)

1.3 Peroxynitrite

Peroxynitrite is an unstable structural isomer of nitrate (NO$_3^-$) with the formula of ONOO$^-$. Despite peroxynitrite being highly reactive as peroxynitrous acid (ONO$^-$OH), it is stable in basic solutions.$^{38}$ In the lab, peroxynitrite standard solution can be made by reacting hydrogen peroxide with sodium nitrite (NaNO$_2$) in aqueous surroundings, which
is stabilized by quickly adding sodium hydroxide (NaOH). Its concentration is determined by measuring the absorbance at 302 nm and using the extinction coefficient (pH 12, \( \varepsilon_{302} = 1670 \text{ M}^{-1} \text{ cm}^{-1} \)).

\[
\text{H}_2\text{O}_2 + \text{NO}_2^- \rightarrow \text{ONOO}^- + \text{H}_2\text{O}
\]  

(1.12)

In physiological surroundings, peroxynitrite is generated from the diffusion-limited reaction between the free radicals of superoxide and nitric oxide.

\[
\cdot \text{NO} + \text{O}_2^- \rightarrow \text{ONOO}^- 
\]

(1.13)

Peroxynitrite is an oxidant with a short lifetime as its highly reactive form of ONOOH. Because of its strongly oxidizing properties, peroxynitrite can damage a wide array of molecules in cells, including DNA and proteins.

Figure 1.4 Isomerization and cleavage of peroxynitrous acid (ONOOH) at low and high concentrations in physiological surroundings. (Modified from\textsuperscript{26})

\[\begin{align*}
\text{H}^+ + \text{NO}_3^- & \rightarrow \text{OH}^- + \text{NO}_2^- \\
\text{Static conditions} & \rightarrow \text{Dynamic conditions}
\end{align*}\]

Heterolytic cleavage  Homolytic cleavage

\[\begin{align*}
\text{ONO}_2^- & \rightarrow \text{OH}^- + \text{NO}_2^+ \\
\text{Isomerization} & \rightarrow \text{Heterolytic cleavage}
\end{align*}\]

\[\begin{align*}
\text{NO}^- & \rightarrow \text{OH}^- + \text{NO}_2^- \\
\text{Homolytic cleavage} & \rightarrow \text{Dynamic conditions}
\end{align*}\]

Figure 1.4 Isomerization and cleavage of peroxynitrous acid (ONOOH) at low and high concentrations in physiological surroundings. (Modified from\textsuperscript{26})

\[\begin{align*}
\text{H}^+ & = \text{proton; NO}_2^- = \text{nitrogen dioxide radical; NO}_2^+ = \text{nitronium ion; NO}_3^- = \text{nitate; OH}^- = \text{hydroxyl radical; OH}^- = \text{hydroxyl ion.}
\end{align*}\]

In static conditions (low concentration), ONOOH can convert to nitrate (NO\textsubscript{3}^-) and proton (H\textsuperscript{+}) via isomerization (Figure 3). However, in dynamic conditions (high
concentration), ONOOH diffusion will speed up due to its concentration gradient between the blood and the cytoplasmic membrane of endothelial cells. During the diffusion process, there are two ways of cleavage that the ONOOH molecule may undergo: heterolytic and homolytic cleavage. The heterolytic cleavage results in the formation of hydroxyl ion (OH⁻) and nitronium ion (NO₂⁺), which is a strong oxidant. The homolytic cleavage of ONOOH produces two radicals: hydroxyl (OH⁻) and nitrogen dioxide (NO₂⁻), which are very strong oxidants as well. O₂⁻, ONOOH, NO₂⁻, OH⁻, and NO₂⁺ are the main constituents involved with oxidative stress, which is usually caused by endothelial dysfunction. Oxidative stress not only results in decreasing the bioavailability of NO, but also leads to cNOS uncoupling, which in return increases the production of O₂⁻ and ONOO⁻. Therefore, peroxynitrite may play an important role in antioxidant enzyme inhibition (such as superoxide dismutase, glutathione reductase), antioxidant depletion, protein aggregation, impairment of enzyme cofactors, membrane channel inhibition, modification of mediator pathways and cellular signaling molecules, upregulation of adhesion receptors, lipid peroxidation, DNA injury and mitochondrial dysfunction.

1.4 Low density lipoprotein

1.4.1 Lipoproteins

Lipoproteins are complex particles with spherical structures where there is a hydrophobic core (including cholesterol esters and triglycerides) surrounded by a hydrophilic shell (consisting of unesterified free cholesterol, phospholipids and apolipoproteins) for each lipoprotein. Apolipoproteins are proteins that bind lipids (such
as fat and cholesterol), which are insoluble in water but vital to cell structure and metabolism, to form the lipoprotein particles which are water-soluble. Then those lipids can be transported around the human body through the bloodstream. There are six classes of apolipoproteins: apo A, B, C, D, E and H, among which there are two major types, apo A and apo B. Apo A can form high-density lipoprotein ("good cholesterol") particles which consist of α-helices and can bind lipid droplets in a reversible way. Apo B is the major component to form low-density lipoprotein ("bad cholesterol") particles, which have a majority of β-sheet structures and are irreversibly bound with lipid droplets. In lipid transportation, those apolipoproteins play an important role as structural components of lipoprotein particles, cofactors for enzymes and ligands for cell-surface receptors in tissues (apo B-100 and apo E for LDL-receptors, apo A-I for HDL receptors).

![Lipoprotein](Modified from68)

Figure 1.5 The structure and components of lipoprotein. (Modified from68)
Due to the difference in fat to protein ratio, lipoproteins may vary in density and particle size. They are larger and less dense when the ratio is high, which indicates there are more fats than proteins, and vice versa. Therefore, based on different density and particle size, lipoproteins are classified as the following (from least dense to most dense in aqueous surroundings): chylomicrons (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL).

Table 1.2 Composition and Density of Human Lipoproteins.\textsuperscript{69}

<table>
<thead>
<tr>
<th>Density (g/mL)</th>
<th>Class</th>
<th>Diameter (nm)</th>
<th>% protein</th>
<th>% cholesterol</th>
<th>% phospholipid</th>
<th>% triacylglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.063-1.210</td>
<td>HDL</td>
<td>5–12</td>
<td>33</td>
<td>30</td>
<td>29</td>
<td>8</td>
</tr>
<tr>
<td>1.019–1.063</td>
<td>LDL</td>
<td>18–25</td>
<td>25</td>
<td>46</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>1.006–1.019</td>
<td>IDL</td>
<td>25–35</td>
<td>18</td>
<td>29</td>
<td>22</td>
<td>31</td>
</tr>
<tr>
<td>0.95–1.006</td>
<td>VLDL</td>
<td>30–80</td>
<td>10</td>
<td>22</td>
<td>18</td>
<td>50</td>
</tr>
<tr>
<td>&lt;0.95</td>
<td>Chylomicron</td>
<td>75-1,200</td>
<td>1-2</td>
<td>8</td>
<td>7</td>
<td>83</td>
</tr>
</tbody>
</table>

1.4.2 Characterization of LDL

LDL is a heterogeneous class of lipoproteins with density between 1.019 and 1.063 g/mL (in aqueous solution) and diameter of 18-25 nm, as well as an average molar mass around 3,000 kDa.\textsuperscript{70} The LDL molecule is globular shaped, consisting of a hydrophobic core that contains triglycerides and about 1500 cholesterol esters on average, surrounded by a hydrophilic surface of phospholipids, free cholesterol, and proteins, mainly apo B-100 (a protein consisting of 4536 amino acids with molar mass of 514 kDa), which acts as a ligand for LDL-specific receptors on the surface of many cells.
to bind the lipoprotein particles, and circulates the fatty acids, making them soluble in the aqueous environment of body fluids.

Cholesterol is taken in daily diet and the body can synthesize cholesterol from various precursors based on demand. Our daily “intake” of cholesterol consists of two parts from distinct resources, about 25% (300 to 500 mg) comes from food, which is called exogenous cholesterol; and the remaining 75% (800 to 1,200 mg) is produced by the body, which is called endogenous cholesterol. Therefore, ingesting cholesterol via daily diet has a limited effect on raising the cholesterol levels in our body. Every cell in the human body can synthesize cholesterol by itself to form cell membranes, therefore only few cells require cholesterol delivery under rare circumstances. Another use of cholesterol is to produce steroid hormones and bile acids.

1.4.3 LDL subtype patterns

It was difficult to characterize LDL structure under physiological conditions due to the heterogeneity of LDL structure. Since the fat to protein ratio varies in LDL particles due to the variable number of fatty acids and proteins, there is a distribution of density and size for LDL particles. Recent research has reported that the structure of LDL particles was determined by using cryo-electron microscopy with a resolution of 16 Angstroms. The heterogeneity in LDL was first proved in the late 1940s. Gofman and colleagues successfully separated the LDL particles into at least three subgroups by using analytical ultracentrifugation. Thereafter, it was further confirmed by density gradient ultracentrifugation (DGUC) and gradient gel electrophoresis (GGE). In general, there are three major types of LDL particles: LDL-I (pattern A) contains majority of
larger and less dense LDL particles with density of 1.025–1.034 g/mL while LDL-III (pattern B) has more of smaller and denser LDL particles with density of 1.044–1.060 g/mL, and LDL-II (pattern I) has density of 1.034-1.044 g/mL. Griffin and colleagues found out that concentration of LDL-III was high in coronary artery disease patients, and it was associated with low concentration of high density lipoprotein (HDL) cholesterol, suggesting that it may be used as a risk marker for coronary artery disease. The conventional way to separate LDL subfractions is carried out by ultracentrifugation with a discontinuous or continuous gradient of potassium bromide (KBr) solution, which is time-consuming (up to 78 hours for sequential flotation or at least 24 hours for separation on discontinuous gradients) and requires overnight-dialysis to remove the salt after ultracentrifugation. Recently, a new method for rapid fractionation of plasma was developed, which involves a new non-ionic, iodinated-density gradient medium, iodixanol. This method is simpler and faster than previous methods, it requires only 3-4 hours ultracentrifugation. The lipoprotein fractions are comparable in density and composition with those separated in conventional salt-based gradients. However, the fractions are slightly less dense than those in salt gradients, due to the fact that the iodixanol gradients are essentially iso-osmotic, keeping protein molecules with their native hydration, as opposed to the dehydration of protein in hyper-osmotic salt gradients, which causes the increase in density.

1.4.4 LDL physiology

LDL cholesterol was once considered as bad cholesterol because it can transport fat and lipids into the wall of arteries, attract macrophages, upregulate the expression of
cell adhesion molecules, and then initiate atherosclerosis, which is the common cause of many cardiovascular diseases. By comparison, HDL cholesterol is often called good cholesterol because it can get rid of extra fat and cholesterol in artery walls.

However, recent studies have shown that small dense LDL may be used as predictors of cardiovascular disease because of its strong association with increased cardiovascular risk.\textsuperscript{79-81} One study showed that women who had cardiovascular issues had higher concentrations of small dense LDL (sdLDLs) particle than that of women who remained free of cardiovascular events. To the contrary, women who remained free of cardiovascular events had higher concentrations of less dense LDL particle than that of women who subsequently had cardiovascular issues.\textsuperscript{82} In addition, several studies have reported that sdLDLs (pattern B LDL) are more susceptible to oxidation than pattern A LDL based on lag time for complete oxidation of LDL particles by using the Esterbauer method.\textsuperscript{83-85} Previous research found that there was a significant correlation ($r=0.46$, $p<0.001$) between oxidative susceptibility and unesterified free cholesterol concentration, which suggests that the oxidative susceptibility of LDL subfractions may be affected by free cholesterol directly.\textsuperscript{86} Furthermore, Chait and colleagues reported that LDL from pattern B was more susceptible to oxidative modification than LDL from pattern A, which may be attributed to the predominance of sdLDLs in pattern B rather than in pattern A LDL.\textsuperscript{87} Once native LDL (n-LDL) turns to oxidized-LDL (ox-LDL), cytotoxicity can ensue by reducing NO production\textsuperscript{88}, increasing oxidative stress\textsuperscript{89} and up-regulating the expression of adhesion molecules,\textsuperscript{90} finally causing the premature development of atherosclerosis.\textsuperscript{91, 92}
The small and dense LDL particles (sdLDLs) are much easier to penetrate the endothelium by going through gaps between endothelial cells (26 nm). After invading the subendothelial space of the vessel wall, sdLDLs will bind to arterial proteoglycans and become susceptible to oxidative modification stimulated by free radicals, which is toxic to the cells. Once ox-LDL formed, which can stimulate the release of growth factors and cytokines, and upregulate the expression of adhesion molecules, attract further monocytes adhering to the surface of endothelium, thus LDL oxidation is an essential cause leading to the development of pro-atherosclerotic lesions. The damage to the artery wall caused by ox-LDL induces the body's immune system to send white blood cells (WBCs), such as macrophages, to engulf the ox-LDL particles by endocytosis via scavenger receptors, which are different from LDL receptors. Those macrophages are not capable of eliminating the ox-LDL. Instead, they become foam cells and then lyse, releasing huge amounts of ox-LDL particles into the artery wall, which in turn attracts more macrophages and causes further foam cells accumulation and smooth muscle cells proliferation, which results in the formation of the plaque.

Infiltration of inflammatory cells, apoptosis of smooth muscle cell, as well as matrix degradation generate a fragile plaque with a thin fibrous cap and a lipid-rich necrotic core. Plaque rupture can cause thrombosis which leads to vessel occlusion. The cholesterol plaque leads to the enlarging of muscle cells and formation of a hard cover over the affected area, which causes narrowing of the artery, reducing the blood flow, increasing blood pressure and finally triggering several cardiovascular disease complications such as atherosclerosis, stroke, hypertension, and heart failure.
1.4.5 Advances in clinical measurement of LDL

Chemical measurement of cholesterol concentration is the most commonly used clinical method because it is more available and less expensive than other methods. In clinical diagnosis, the concentration of LDL particles is not measured directly. Instead, Friedewald equation is used to estimate low density lipoprotein cholesterol (LDL-C) by subtracting cholesterol amount in HDL and VLDL from the total amount of cholesterol. This method is commonly used in blood test reports to estimate how many LDL cholesterol particles are driving the progression of atherosclerosis. However, there are some limitations to this method, such as calculation of LDL-C becomes inaccurate if the concentration of plasma triglyceride is over 4.52 mmol/L (400 mg/dL). Even if triglyceride concentration is at the ideal range of 2.5 to 4.5 mmol/L, this equation is still considered inaccurate. Furthermore, this method cannot get the accurate concentration of LDL particle because the percentage of cholesterol varies within different LDL particles due to its heterogeneity, leading to the fact that LDL-C values are usually inconsistent with measuring both LDL particles and the actual development rates of atherosclerosis directly, which makes it difficult to evaluate the progression status of atherosclerosis based on LDL-C values. It turns out that many people who are considered as “low risk” according to the LDL-C values have suffered from CVD events.

There are several methods for measuring LDL particle concentrations and size. Direct measurements of LDL particle concentration such as nuclear magnetic resonance (NMR) can reveal clinical outcome better than LDL-C evaluation method, but they are not as widely used as LDL-C due to high costs and hardware requirement, which make it
available only in a handful of laboratories in the United States. With the help of NMR technology, which was pioneered by Jim Otvos and colleagues,\textsuperscript{82,100,101} there is a 22-25% decrease in CVD events within only one year.\textsuperscript{102} However, the NMR test is still less widely available and more expensive than other competing methods. Since the later 1990s, NMR measurements were developed greatly to decrease costs and improve precision; it became possible to measure lipoprotein particles in clinics at lower cost (< $100 compared to the previous costs from $400 to $5,000) and higher accurateness. There are also other homogeneous methods for LDL particle measurement, however, most of them only get the LDL particle concentration by estimation. In 2008, the American Diabetes Association (ADA) and the American College of Cardiology Foundation (ACCF) recommended direct measurement of LDL particle numbers by NMR as the superior way for evaluating individual risk of cardiovascular diseases, due to its advantages for predicting progression of atherosclerosis diseases.\textsuperscript{103}

In addition, the density of LDL particles and their size distribution, in association with their concentration, have a stronger correlation and more consistent with atherosclerotic progression and cardiovascular events than LDL-C values.\textsuperscript{104} It is possible that the concentration of LDL cholesterol is at the regular level which is considered as healthy, while small and dense LDL particle number is high and cardiovascular events risks are high. Similarly, it is very likely that LDL cholesterol concentration is slightly higher than normal range, yet LDL particle number is low and cardiovascular events risks are also low. In the United States, the American Heart Association (AHA) and the
National Institutes of Health (NIH) provide several guidelines for interpreting LDL-C levels and risk rate for CVD events.\textsuperscript{105}

Table 1.3 Classification and risk interpretation of different LDL cholesterol level.\textsuperscript{105}

<table>
<thead>
<tr>
<th>Level (mg/dL)</th>
<th>Level (mmol/L)</th>
<th>Risk Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 to &lt;50</td>
<td>&lt;1.3</td>
<td>Optimal LDL cholesterol, levels in healthy young children before onset of atherosclerotic plaque in heart artery walls</td>
</tr>
<tr>
<td>&lt;70</td>
<td>&lt;1.8</td>
<td>Optimal LDL cholesterol, corresponding to lower rates of progression, promoted as a target option for those known to clearly have advanced symptomatic cardiovascular disease</td>
</tr>
<tr>
<td>&lt;100</td>
<td>&lt;2.6</td>
<td>Optimal LDL cholesterol, corresponding to lower, but not zero, rates for symptomatic cardiovascular disease events</td>
</tr>
<tr>
<td>100 to 129</td>
<td>2.6 to 3.3</td>
<td>Near optimal LDL level, corresponding to higher rates for developing symptomatic cardiovascular disease events</td>
</tr>
<tr>
<td>130 to 159</td>
<td>3.3 to 4.1</td>
<td>Borderline high LDL level, corresponding to even higher rates for developing symptomatic cardiovascular disease events</td>
</tr>
<tr>
<td>160 to 199</td>
<td>4.1 to 4.9</td>
<td>High LDL level, corresponding to much higher rates for developing symptomatic cardiovascular disease events</td>
</tr>
<tr>
<td>&gt;200</td>
<td>&gt;4.9</td>
<td>Very high LDL level, corresponding to highest increased rates of symptomatic cardiovascular disease events</td>
</tr>
</tbody>
</table>

1.5 Endothelial dysfunction

The endothelium functions as a mediator of relaxations or contractions of arteries, blood vessels and veins in the cardiovascular system.\textsuperscript{106} Endothelium mediates those responses through several substances, such as nitric oxide (also known as EDRF), endothelium-derived hyperpolarizing factor (EDHF), prostacyclin and some other contracting factors.\textsuperscript{107, 108} There are many factors leading to endothelium dysfunction, including, but not limited to, old age, smoking, obesity, increased cholesterol level in
serum, and family history of CVD. In addition, eNOS uncoupling can also cause endothelial dysfunction. Under functional conditions, eNOS can synthesize endothelial NO from L-arginine; while under dysfunctional status, eNOS is uncoupled and switches from producing NO (as its oxygenase function) to producing ONOO\(^-\) (as its reductase function) by catalyzing the reaction between NO and O\(_2^-\).

Deficiency of BH\(_4\) and/or L-arginine facilitates eNOS uncoupling and generates much more O\(_2^-\) than normal functional endothelium. The reaction between NO and O\(_2^-\) is in a rapid, diffusion-controlled way, resulting in reduction of bioavailability of NO and increased oxidative stress.\(^{109}\) Endothelial dysfunction leads to reduced bioavailability of NO, elevated level of adhesion molecule expression, increased release of chemokine and other cytokines, as well as reactive oxygen species (ROS) production from the endothelium.\(^{110}\) Endothelial dysfunction is considered as a pivotal early event in the development of atherosclerosis\(^{111}\) and is associated with many CVDs, such as stroke, hypertension, heart failure and coronary artery disease, not only because it contributes to inflammation and myofibroblast migration, but also causes smooth muscle cell proliferation inside the vessel, all of which leads to the progression of atherosclerosis.

### 1.6 Research significance and goals

LDL cholesterol has been considered as bad cholesterol because it transports lipids and fats to arteries, which may lead to cardiovascular diseases. Therefore, small dense LDL levels have been used to estimate risk for cardiovascular disease.\(^{79, 80, 112}\) The heterogeneity of LDL was confirmed by several studies, suggesting that there are several subtype patterns of LDL with different densities.\(^{78, 113, 114}\) In general, there are three major
types of LDL particles: LDL-I (pattern A), LDL-II (pattern I) and LDL-III (pattern B). Although it’s possible that pattern B LDL particles carry the same cholesterol content as pattern A LDL, pattern B LDL is considered as a higher risk factor for CVD than Pattern A, not only because pattern B LDL can induce much faster growth of atheroma (i.e., progression of atherosclerosis), but also causes much more severe cardiovascular diseases. Another possible reason is that the small and dense LDL particles are much easier to penetrate the endothelium, where pattern B LDL is more susceptible to be oxidized than pattern A LDL, and may potentially cause a dysfunction of endothelium and change the balance between cytoprotective nitric oxide and cytotoxic peroxynitrite. This processes can be affected by age, environmental factors and/or ethnic.

African Americans are believed to have higher risk than Caucasian Americans for developing cardiovascular diseases, such as stroke, hypertension and heart failure. Previous studies have shown that African Americans have inherent imbalance of nitric oxide (NO) and peroxynitrite (ONOO⁻) production in endothelium. NO is not only a physiological mediator and signaling molecule, but also plays an important role in inhibiting smooth muscle cell proliferation, adhesion of leukocytes to the endothelial cells, and platelet aggregation. Reduced bioavailability of NO and overproduction of ONOO⁻ can cause the release of cytotoxic radicals and endothelium dysfunction, which may explain the higher rates of cardiovascular morbidity and mortality in African Americans compared to Caucasian Americans. Endothelial dysfunction induced by LDL is related with many forms of CVDs, such as atherosclerosis, hypertension, coronary
artery disease,\textsuperscript{91} diabetes\textsuperscript{123} and peripheral artery disease.\textsuperscript{124} Endothelial cell dysfunction induced by protracted exposure of high concentration of LDL may take place first, and then is followed by LDL accumulation in the subendothelial space.\textsuperscript{125} It has been proved that n-LDL and ox-LDL may cause uncoupling of eNOS, resulting in superoxide and peroxynitrite production in endothelial cells.\textsuperscript{88} Furthermore, ox-LDL may enhance endothelial damage by inducing foam cell generation and smooth muscle proliferation, leading to the progression of atherosclerosis.\textsuperscript{92} Ox-LDL can also inhibit endothelium dependent relaxation by decreasing NO production\textsuperscript{88} and reduce the anti-aggregating properties of the endothelial.\textsuperscript{126}

However, the effect of different patterns of LDL in the cardiovascular system, especially in endothelial cells, has not been fully elucidated yet. In this study, we hypothesize that different subpatterns of LDL may differently influence the kinetics of NO release from endothelium. And as an oxidation/nitrooxidation stress, ONOO$^-$ production can be strongly influenced by the kinetics of NO production. Therefore, different subpatterns of LDL may generate different levels of oxidative stress; which may affect the function of endothelium; increasing ICAM-1 and VCAM-1 expression, monocyte adhesion, and formation of atherosclerotic lesion. A quantification of NO release, as a function of LDL density and/or LDL subpatterns distribution, may be used for early diagnosis of cardiovascular disease. We used a well-established method (electrochemical nanosensors) to measure the real-time release of the NO and ONOO$^-$ from cultured endothelial cells stimulated by different LDL subpatterns. We hypothesized that different LDL subpatterns can induce different ONOO$^-$ and NO
concentration, which may result in shift unfavorable NO/ONOO⁻ balance and cause the upregulation of monocyte adhesion.

Another goal in this study is to investigate the correlation of race-specific differences in endothelial function in the presence of LDL with different patterns. We hypothesize that endothelial cells of African Americans may be more affected by LDL stimulation than endothelial cells of Caucasian Americans. Small and dense LDL may cause unfavorable imbalance of the NO and ONOO⁻ ratio, which may be more significant in Blacks than Whites. Native and oxidized LDL (n-LDL and ox-LDL) with different patterns will be used to stimulate endothelium to release NO and ONOO⁻, as well as inducing upregulation of the expression of cell adhesion molecules (CAMs) and monocyte adhesion, which may reveal the role of LDL subpatterns in induction of NO and ONOO⁻ in CVD and shed light on the mechanism of sdLDL involved in race-specific development of atherosclerotic CVD. Finally, this research will propose a parameter-based model for more precise estimation of the LDL induced cardiovascular risk and higher rate of cardiovascular morbidity and mortality in African Americans compared to Caucasian Americans.
2 Materials and methods

2.1 Cell cultures

Human umbilical vein endothelial cells (HUVECs) and human monocytoid cells (THP-1) were purchased from the American Type Culture Collection (ATCC). HUVECs were cultured as monolayer in MCDB-131 Complete Medium (VEC tech) at 37°C in a humidified atmosphere enriched with 5% CO₂. The THP-1 cells were cultured in RPMI-1640 medium (ATCC) containing 10% FBS (ATCC), 100 U/mL penicillin (ATCC) and 100 U/mL streptomycin (ATCC) at 37°C in a humidified atmosphere enriched with 5% CO₂.

2.2 Electrochemical measurements for NO and ONOO⁻

2.2.1 Principles of NO detection

Concurrent measurements of NO were performed with electrochemical nano sensors. The designs of nanosensors are based on previously well-developed chemically modified carbon-fiber technology. Electrochemical oxidation of NO on such a nano electrode was used to detect its concentration. The current generated from electron transfer via NO oxidation was proportional to the concentration of NO and measurements were carried out in amperometric mode in which the potential was kept constant between the nanosensor (working electrode) and the reference electrode. A response time of NO nanosensor is better than 1 ms and detection limit is $1 \times 10^{-9}$ mol/L. NO was measured by using a three-electrode system consisting of a NO-sensitive nanosensor (working electrode), a counter electrode made of platinum wire and a silver/silver chloride (Ag/AgCl) reference electrode (SSCE). The measurements were performed at a constant
potential of 700 mV vs. Ag/AgCl utilizing a Gamry Reference 600 potentiostat (Gamry Instrument, Warminster, PA) connected to computer with Gamry VFP 600 software (Gamry Instrument, Warminster, PA).

2.2.2 Preparation of NO nanosensor

Each nanosensor for NO was fabricated by placing a carbon fiber into an open-ended glass capillary which was pre-heated in a Bunsen burner flame to form an opening trip at one end with diameter of 10-15 µm. (Figure 2.1) Then 6-cm-long straightened copper wire was prepared by cutting copper wire into the designated length and polished the whole length with emery paper to reduce the internal resistance and thereby increase its sensitivity. Silver epoxy was made by mixing parts A and B from conductive silver epoxy kit (Epoxy Technology, Billerica, MA) in an equal amount and later was used to coat the surface of polished copper wire. Once copper wire was coated with silver epoxy, it was inserted into the glass capillary and heated in a vacuum dryer at 40 ºC for 3 hours to provide electrical contact between copper wire and carbon fiber. Molten beeswax was used to seal the interstitial space between tapered glass tip and carbon fibers. Extra beeswax from outside of glass tip was removed by immersing the assembled module in 0.1 mol/L NaOH solution overnight.

Before coating the protruding carbon fibers with porphyrin, NO electrodes underwent electrochemical pretreatment by scanning the potential at ±1.50 V for two cycles (scan rate at 100 mV/s) in 0.1 mol/L NaOH solution (saturated with nitrogen for 15 minutes). Then the carbon fiber was coated electrochemically with a conductive film of polymeric nickel (II) tetrakis (3-methoxy-4-hydroxy-phenyl) porphyrinic (Ni-TMHP)
(Frontier Scientific). The porphyrinic film was deposited by scanning potential between 0.00 V and 1.00 V at scan rate of 100 mV/s for 15-25 cycles. (Figure 2.9) After deposition of porphyrinic film on the carbon fibers, NO sensors were immersed into 5% (v/v in methanol) Nafion solution (Aldrich, USA) for 10 seconds and allowed to dry in the air for two minutes, then the process was repeated three times. Negatively charged Nafion layer attracts NO$^+$ (product of NO oxidation) while expelling NO$_2^-$ and other anions, which may be oxidized at 700 mV. Finally NO nanosensors were stored in small glass vials filled with double-distilled water at room temperature.$^{16}$

Figure 2.1 The schematic diagram of NO electrochemical nanosensors. (Adapted from$^{128}$)
Figure 2.2 Multiple-scan cyclic voltammograms for electrochemical deposition of a polymeric Ni-TMHPP on the tip of the carbon fibers. Peak $I_O$ indicates oxidation of Ni (II) to Ni (III) and peak $I_R$ indicates reduction of Ni (III) to Ni (II). Rising current ($I_a$) indicates catalytic oxidation of $H_2O$.

### 2.2.3 Calibration of NO nanosensor

NO standard stock solution was prepared according to the following reaction. Experimental set-up for synthesis of NO is shown in figure 2.3.

\[
6 \text{NaNO}_2 + 3 \text{H}_2\text{SO}_4 \rightarrow 4 \text{NO} + 2 \text{HNO}_3 + 2 \text{H}_2\text{O} + 3 \text{Na}_2\text{SO}_4
\]  
(2.1)

NO generated from the reaction above was bubbled in a nitrogen gas atmosphere through 4 mol/L and 2 mol/L NaOH solutions, and finally dissolved in deoxygenated phosphate buffer solution (pH 7.4). The actual concentration of NO standard solution was confirmed by spectrophotometer as described previously.\textsuperscript{129} Calibration was applied to each NO nanosensor by injecting NO standard phosphate buffer solution with different
volumes. Linear calibration curves were constructed for each nanosensor based on the current responses (from peak to its basal line) to different NO concentrations by amperometry with time (detection limit of 1 nmol/L and resolution time <50 ms for each nanosensor).

Figure 2.3 Experimental setup for synthesis of NO standard solution. (Adapted from130)

Calibration was applied to each NO nanosensor by injecting NO standard phosphate buffer solution with different volumes. Linear calibration curves were constructed for each nanosensor based on the current responses (from peak to its basal line) to different concentrations of NO standard by amperometry with time.
2.2.4 Principles of ONOO⁻ detection

Concurrent measurements of ONOO⁻ were performed with electrochemical nano sensors. The designs of nano sensors are based on previously described nanosensor with several modifications.\textsuperscript{131} Electrochemical reduction of ONOO⁻ on such a nanosensor was used to detect its concentration. The current generated from electron transfer via ONOO⁻ reduction was proportional to the concentration of ONOO⁻ and measurements were carried out in amperometric mode in which the potential was kept constant between the nanosensor (working electrode) and the reference electrode. Measurement was performed by using three-electrode system consisting of a ONOO⁻-nanosensor (working electrode), a counter electrode made of platinum wire and a Ag/AgCl reference electrode (SCE). The
measurements were performed at a constant potential of -450 mV vs. Ag/AgCl utilizing a Gamry Reference 600 potentiostat connected to computer with Gamry VFP 600 software.

2.2.5 Preparation of ONOO$^-$ nanosensor

Preparation of ONOO$^-$ nanosensor is based on previously described method with significant modification,$^{131,132}$ which is quite similar to NO nanosensor. (Figure 2.6) A conductive polymeric film was deposited electrochemically with Mn (III)–paracyclophanyl-porphyrin (Frontier Scientific) at scanning potential between -0.50 V and 1.00 V at scan rate of 100 mV/s (30 cycles). After deposition of porphyrin on the carbon fiber, ONOO$^-$ sensors were taken out from the coating solution and rinsed with acetone then distilled water, and allowed to dry in the air. Those nanosensors were immersed into 1% (w/v in methanol) poly (4-vinylpyridine) solution (Aldrich, USA) for 10 seconds and allowed to dry in the air for two minutes. The coating layer of poly (4-vinylpyridine) provides a barrier against positively charged molecules such as proteins. The nanosensors were then placed in phosphate buffer solution and cyclic voltammetry was applied between -0.20 and +0.10 V at scan rate of 100 mV/s for about 13 cycles. Finally ONOO$^-$ nanosensors were stored in small glass vials filled with double-distilled water at room temperature.
Figure 2.5 The schematic diagram of ONOO$^-$ electrochemical nanosensors\textsuperscript{131}

Figure 2.6 Multiple-scan cyclic voltammograms for electrochemical deposition on the tip of carbon fibers. Peak $I_O$ indicates oxidation of Mn (III) to Mn (IV) and peak $I_R$ indicates reduction of Mn (IV) to Mn (III).
2.2.6 Calibration of ONOO⁻ nanosensor

ONOO⁻ standard stock solution was prepared according to the previously described procedure with some modifications. The experimental set-up for synthesis of ONOO⁻ standard solution was performed in an Erlenmeyer flask connected with a system of rubber tubing and T-junctions as shown in Figure 2.7. Both of 0.6 mol/L NaNO₂ solution and 0.6 mol/L HCl/0.7 mol/L H₂O₂ solution were pumped into the T-junction and mixed in a glass tube. The acid-catalyzed reaction of nitrite with H₂O₂ to form peroxynitrous acid was quenched by 0.9 mol/L NaOH solution, and the excess H₂O₂ was removed by treating with MnO₂ powder. Peroxynitrite solution showed a strong yellow color and was later directed into collection flask. All solutions were freshly made before synthesis of ONOO⁻ standard solution and frozen to 0 °C by keeping those flasks in ice-bath Styrofoam containers. The standard solution was immediately transferred to Eppendorf tubes wrapped with aluminum foil and then stored at -80 °C for future use. Synthesis of peroxynitrite is based on these equations:

\[
\text{NaNO}_2 + \text{HCl} + \text{H}_2\text{O}_2 \rightarrow \text{ONOOH} + \text{H}_2\text{O} + \text{NaCl} \quad (2.2)
\]

\[
\text{ONOOH} + \text{OH}^- \rightarrow \text{ONOO}^- + \text{H}_2\text{O} \quad (2.3)
\]

The concentration of ONOO⁻ standard solution was determined by using spectrophotometer (Beckman Coulter DU 640, UV-Vis, λ\text{max} = 302 nm). Calibration was applied to each ONOO⁻ nanosensor by injecting ONOO⁻ standard solution with different volumes. Linear calibration curves were constructed for each nanosensor based on the current responses (from peak to its basal line) to different ONOO⁻ concentrations by amperometry with time.
Figure 2.7 Experimental setup for synthesis of ONOO\(^-\) standard (adapted from \(^{134}\)).

Calibration was applied to each ONOO\(^-\) nanosensor by injecting ONOO\(^-\) standard solution with different volumes. Linear calibration curves were constructed for each nanosensor based on the current responses (from peak to its basal line) to different concentrations of ONOO\(^-\) standard by amperometry with time.

Figure 2.8 The standard calibration curve of ONOO\(^-\) nanosensor (R\(^2\)=0.9977, n=3).
2.3 Isolation, oxidation and analysis of LDL

2.3.1 LDL subpatterns isolation and oxidation

Normal human plasma (11 mL, Innovative Research) was mixed with 11mL of iodixanol (OptiPrep density gradient medium, Sigma) to make a final concentration of iodixanol of 6% (w/v). The mixture was loaded to the OptiSeal centrifuge tube (volume 11.2 mL, Beckman Coulter) and placed in NVT65 rotor (Beckman Coulter), then centrifuged at 60,000 rpm (342,000 g) for 4 hours at 16°C in Optima L-90K ultracentrifuge (Beckman Coulter) set at slow acceleration and slow deceleration. Samples were fractionated within 1 hour after centrifugation. Fractions were collected from each gradient by downward displacement using a syringe tip pierced the bottom of the tube and pumped out. The fractions were collected into Eppendorf tubes with 1.5 ml per fraction. Oxidized-LDL (ox-LDL) was prepared according to previous reported method.\textsuperscript{88} CuSO\textsubscript{4} was added to native LDL (n-LDL) with final concentration of 10 µmol/L. Oxidation was carried out at room temperature over 24 hours until oxidation was complete. The ox-LDL was then placed in ultra-centrifuge tubes (Sigma-Aldrich, Ultra-4, MWCO 30 kDa) and centrifuged at 3000 rpm for 20 minutes to remove CuSO\textsubscript{4}. All of the LDL samples were filtered and stored at 4°C for future use.
2.3.2 LDL analysis

The density of LDL sample fractions was measured indirectly by using pocket refractometer (ATAGO). In brief, the prism surface was cleaned by Kimwipes and one drop of distilled water was placed to the surface to set up the zero value. Then 120 µL of LDL sample was loaded to the prism surface and the refractive index was recorded, which was later converted to density by using the following equation.\(^\text{135}\)

\[
\rho = 3.3623\eta - 3.4837
\]  

(2.4)

Where \(\rho\) is the density of LDL sample with unit of g/mL, and \(\eta\) is the refractive index.

The concentration of LDL sample fractions was measured by using the Amplex® Red cholesterol assay kit (Invitrogen). A cholesterol standard curve was made by measuring the fluorescence intensity of gradient concentrations of cholesterol reference standard. LDL samples were pre-diluted to proper concentration within the linear range.
of detection, and the cholesteryl esters were digested by cholesterol esterase to free cholesterol, which was then detected in the enzyme-coupled reaction with Amplex® Red reagent. Finally, the fluorescence intensity was obtained from multi-detection microplate reader (Synergy HT, BioTek) and converted to concentrations based on standard curve.

2.4 Determination of n-LDL/ox-LDL induced NO and ONOO⁻ release from endothelial cells

Endothelial cells were seeded to 24 well plates and cultured in complete medium until a confluent monolayer formed. Then the study was carried out as follows: (a)

Endothelial cells were stimulated with direct injection of n-LDL with different densities (pattern A: 1.016-1.019 g/mL, pattern I: 1.024-1.029 g/mL, and pattern B: 1.034-1.053 g/mL) and different concentrations (50, 100, 250, 500, 750, 1000 µg/mL), and the release of NO/ONOO⁻ was measured by placing a NO/ONOO⁻ nanosensor on the surface of endothelial cells to collect the electric signal from the active tip of NO/ONOO⁻ nanosensor. (b) Endothelial cells were stimulated with direct injection of n-LDL with different mixture combinations of pattern A, B and I LDL (800 µg/mL) as following: (1) 60% A, 20% B and 20% I; (2) 20% A, 60% B and 20% I; (3) 20% A, 20% B and 60% I; (4) 50% A and 50% B; (5) 50% A and 50% I; (6) 50% B and 50% I; (7) 33% A, 38% B and 29% I (simulation of original constituent from general human plasma), and the release of NO/ONOO⁻ was measured in the same way as described above. (c) Endothelial cells were pre-treated with polyethylene glycol covalently linked to superoxide dismutase (PEG-SOD, 400 U/mL), L-arginine (300 µmol/L), a precursor of constitutive nitric oxide synthase (cNOS) cofactor tetrahydrobiopterin (sepiapterin, 200 µmol/L), L-nitro-arginine
methyl ester (L-NAME, 100 µmol/L) and a selective nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor (VAS2870, 10 µmol/L) in endothelial basal medium (EBM) at 37°C for 30 minutes, control group was incubated in EBM only. After incubation, endothelial cells were stimulated with direct injection of pattern A, B and I n-LDL (800 µg/mL), and the release of NO/ONOO\(^{-}\) was measured in the same way as described above. (d) Endothelial cells were stimulated with direct injection of n-LDL/ox-LDL (800 µg/mL) and the release of NO/ONOO\(^{-}\) was measured in the same way as described above.

HUVECs from African American (African Americans) and Caucasian (Caucasian Americans) were seeded to 24 well plates and cultured in complete medium until confluent monolayer formed. Then the study was carried out as follows: (a) Endothelial cells were stimulated with direct injection of n-LDL (800 µg/mL) with different densities (pattern A: 1.016-1.019 g/mL, pattern I: 1.024-1.029 g/mL, and pattern B: 1.034-1.053 g/mL), and the release of NO/ONOO\(^{-}\) was measured by placing a NO/ONOO\(^{-}\) nano sensor on the surface of endothelial cells to collect the electric signal from the active tip of NO/ONOO\(^{-}\) nanosensor. (b) Endothelial cells were stimulated with direct injection of ox-LDL (800 µg/mL) with different densities (pattern A: 1.016-1.019 g/mL, pattern I: 1.024-1.029 g/mL, and pattern B: 1.034-1.053 g/mL), and the release of NO/ONOO\(^{-}\) was measured in the same way as described above. (c) Endothelial cells were stimulated with direct injection of n-LDL with different mixture combinations of pattern A, B and I LDL (800 µg/mL) as following: (1) 60% A, 20% B and 20% I; (2) 20% A, 60% B and 20% I; (3) 20% A, 20% B and 60% I; (4) 50% A and 50% B; (5) 50% A and 50% I; (6) 50% B
and 50% I; (7) 33% A, 38% B and 29% I (combination from original LDL), and the release of NO/ONOO− was measured in the same way as described above.

2.5 Cell ELISA

Cell ELISA was used to detect the expression of adhesion molecules. Endothelial cells were seeded in 96 well plates with complete medium until a confluent monolayer formed. Then cells were incubated with n-LDL/ox-LDL (400 μg/mL) at 37°C for 5 hours. Control is EBM with 3% (w/v) iodixanol. After stimulation with LDL, endothelial cells were washed with phosphate buffered saline (PBS) twice and fixed with 4% (w/v) formaldehyde solution for 20 minutes at room temperature. After fixation, HUVECs were washed twice with phosphate buffered saline with tween-20 (PBST). Afterwards the cells were incubated with blocking buffer (4% BSA in PBST) for 1 hour at room temperature. After blocking, the plate was washed three times with PBST. Primary monoclonal antibody against ICAM-1 and VCAM-1 (Santa Cruz) diluted in PBST (0.5 μg/mL for ICAM-1, and 2 μg/mL for VCAM-1) were added to the cells at 4°C overnight. After incubation, the plate was washed three times with PBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz) diluted at 1:1000 in PBST for 1 hour at room temperature. The cells were washed again three times, and 4,4'-Bi-2,6-xylidine;4,4'-Diamino-3,3',5,5'-tetramethylbiphenyl (TMB) solution was added to each well and incubated at room temperature. Then, 2 mol/L citric acid solution was added to each well. The absorbance was measured at 450 nm in a microplate reader. Each experiment was performed in six duplicates and repeated at least three times.
Endothelial cells (African American and Caucasian) were seeded in 96 well plates with complete medium until confluent monolayer formed. Then cells were incubated with n-LDL/ox-LDL (400 μg/mL) at 37°C for 5 hours. Control is EBM with 3% (w/v) iodixanol. After stimulation with LDL, endothelial cells were washed with PBS twice and fixed with 4% (w/v) formaldehyde solution for 20 minutes at room temperature. After fixation, HUVECs were washed twice with phosphate buffered saline with tween-20 (PBST). Afterwards the cells were incubated with blocking buffer (4% BSA in PBST) for one hour at room temperature. After blocking, the plate was washed three times with PBST. Primary monoclonal antibody against ICAM-1 and VCAM-1 diluted in PBST (0.5 μg/mL for ICAM-1, and 2 μg/mL for VCAM-1) were added to the cells at 4°C overnight. After incubation, the plate was washed three times with PBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG diluted at 1:1000 in PBST for 1 hour at room temperature. The cells were washed again three times, and TMB solution was added to each well and incubated at room temperature. Then, 2 mol/L citric acid solution was added to each well. The absorbance was measured at 450 nm in a microplate reader. Each experiment was performed in six duplicates and repeated at least three times.

2.6 Monocyte adhesion

Endothelial cells were seeded in 96 well plates with complete medium until confluent monolayer formed. THP-1 cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. THP-1 cells were pre-labeled with 2’, 7’-bis-(2-
carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Molecular Probes, Life technology) for quantitative adhesion assay. Fluorescence labeling of THP-1 cells was carried out by incubating cells \(5 \times 10^6\) cells/mL with 5 \(\mu\)mol/L BCECF-AM in RPMI-1640 medium for 30 minutes at 37°C. After incubation, cells were washed three times with PBS to remove excess dye. Cells were then re-suspended in EBM at a density of 10\(^6\) cells/mL. Then the study was carried out as follows: (1) Confluent HUVECs were incubated with constant concentration (400 \(\mu\)g/mL) of n-LDL at 37°C for 5 hours. Then cells were washed with PBS twice to remove LDL. Fluorescently labeled THP-1 cells were added to the surface of confluent endothelial monolayer as \(5 \times 10^4\)/well and co-incubated at different time intervals (from 10 to 60 minutes); and then the co-cultured THP-1 cells were washed twice with PBS in order to eliminate the non-adherent cells. The fluorescence intensity of each well was measured by using a fluorescence multi-well plate reader set at excitation and emission wavelengths of 485 and 528 nm, respectively. (2) Confluent endothelial cells were incubated with LDL at a final concentration of 50, 100, 200 and 400 \(\mu\)g/mL at 37°C for 5 hours. Then cells were washed with PBS twice to remove LDL. Fluorescently labeled THP-1 cells were added to the surface of confluent endothelial monolayer as \(5 \times 10^4\)/well and co-incubated at 37°C for 1 hour; and then the co-cultured THP-1 cells were washed twice with PBS in order to eliminate the non-adherent cells. The fluorescence intensity of each well was measured in the same way as described above. (3) Confluent endothelial cells were incubated with n-LDL/ox-LDL (400 \(\mu\)g/mL) at 37°C for 5 hours. Then cells were washed with PBS twice to remove LDL. Fluorescently labeled THP-1 cells were
added to the surface of confluent endothelial monolayer as $10^5$/well and co-incubated at 37°C for 1 hour; and then the co-cultured THP-1 cells were washed twice with PBS in order to eliminate the non-adherent cells. The fluorescence intensity of each well was measured in the same way as described above.

Endothelial cells (African Americans and Caucasian Americans) were seeded in 96 well plates with complete medium until confluent monolayer formed. THP-1 cells were pre-labeled with BCECF-AM for quantitative adhesion assay. Fluorescence labeling of THP-1 cells was carried out by incubating cells ($5 \times 10^6$ cells/mL) with 5μmol/L BCECF-AM in RPMI-1640 medium for 30 minutes at 37°C. After incubation, cells were washed three times with PBS to remove excess dye. Cells were then re-suspended in EBM at a density of $10^6$ cells/mL. Then the study was carried out as follows: (1) Confluent endothelial cells were incubated with n-LDL (400 μg/mL) at 37°C for 5 hours. Then cells were washed with PBS twice to remove LDL. Fluorescently labeled THP-1 cells were added to the surface of confluent endothelial monolayer as $10^5$/well and co-incubated at 37°C for 1 hour; and then the co-cultured THP-1 cells were washed twice with PBS in order to eliminate the non-adherent cells. The fluorescence intensity of each well was measured by using a fluorescence multi-well plate reader set at excitation and emission wavelengths of 485 and 528 nm, respectively. (2) Confluent endothelial cells were incubated with ox-LDL (400 μg/mL) at 37°C for 5 hours. Then cells were washed with PBS twice to remove LDL. Fluorescently labeled THP-1 cells were added to the surface of confluent endothelial monolayer as $10^5$/well and co-incubated at 37°C for 1 hour; and then the co-cultured THP-1 cells were washed twice with PBS in order to
eliminate the non-adherent cells. The fluorescence intensity of each well was measured as described above.

![Diagram](image)

Figure 2.10 Schematic diagram for monocyte adhesion assay.

2.7 Statistical analysis

All data are based on at least 3 independent measurements and expressed as mean±SD. Student's t-test was used to analyze the significance of data. A value of P<0.01 was considered significant.
3 Results

3.1 Isolation, oxidation and analysis of LDL

The density profile generated by iodixanol gradient solution in four independent separation experiments is shown in Figure 3.1. The density gradient from whole plasma fractions ranges from 1.010-1.176 g/mL, among which LDL samples range from 1.018 to 1.048 g/mL. In general, we can classify these LDL subfractions into three subfraction groups, which are fractions 3-4 (1.034-1.048 g/mL) as pattern B LDL, fraction 5 (1.026 g/mL) as pattern I LDL, and fraction 6 (1.018 g/mL) as pattern A LDL.

![Density profile generated by iodixanol gradient solution after 4 hours ultracentrifugation (n=4)](image)

Figure 3.1 Density profile generated by iodixanol gradient solution after 4 hours ultracentrifugation (n=4)

A Cholesterol standard curve was made by measuring the fluorescence intensity of gradient concentrations of cholesterol reference standard. Cholesterol reference was
detected in the enzyme-coupled reaction with Amplex® Red reagent. Finally, the fluorescence intensity was obtained from multi-detection microplate reader.

![Figure 3.2 Cholesterol standard curve (R²=0.9944, n=3).](image)

**3.2 Determination of n-LDL/ox-LDL induced NO and ONOO⁻ release from endothelial cells**

**3.2.1 n-LDL induced NO and ONOO⁻ release in endothelial cells**

To determine the biological availability of NO and ONOO⁻ after LDL induction, we measured the real-time production of NO and ONOO⁻ in LDL-stimulated HUVECs by using NO/ONOO⁻ nanosensors. By positioning the nanosensors near the surface of endothelial cells (5 ± 2 μm), the productions of NO/ONOO⁻ were measured after direct injection of LDL. To determine the dose effect of LDL induced HUVECs, we measured the real-time production of NO and ONOO⁻ after direct injection of LDL with different...
density and concentration. Our data showed that a rapid release of NO / ONOO\(^-\) was detected after less than 0.1 s after direct injection of LDL, and the maximal [NO] and [ONOO\(^-\)] were reached about 1.0 s after LDL injection (Figure 3.3, 3.4).

Figure 3.3 Amperograms of NO release from endothelial cells stimulated with direct LDL injection (LDL of Pattern A, B and I, 1000 µg/mL).
Figure 3.4 Amperograms of ONOO⁻ release from endothelial cells stimulated with direct LDL injection (LDL of Pattern A, B and I, 1000 µg/mL).

At the maximal concentration (1000 µg/mL) of LDL direct injection, pattern A LDL can stimulate endothelial cells to produce the highest concentration of NO (591±73 nmol/L) and lowest concentration of ONOO⁻ (222±23 nmol/L), while pattern B LDL can stimulate endothelial cells to produce the lowest concentration of NO (211±18 nmol/L) and highest concentration of ONOO⁻ (409±36 nmol/L). Pattern I LDL stimulates endothelial cells to produce NO at 300±28 nmol/L and ONOO⁻ at 323±29 nmol/L. (Figure 3.5).
Figure 3.5 Maximal [NO] and [ONOO⁻] released from endothelial cells after LDL injection (LDL of Pattern A, I and B, 1000 µg/mL). NO (solid bars) and ONOO⁻(open bars). Data are expressed as mean±SD. *P<0.01 vs control (n=4).

A ratio of [NO] to [ONOO⁻] was used to indicate the oxidative stress level and NO / ONOO⁻ imbalance in endothelial cells. (Figure 3.6). High ratio of [NO] to [ONOO⁻] indicates a high level of NO bioavailability and/or relative low concentration of cytotoxic ONOO⁻. The ratio of [NO] to [ONOO⁻] was 0.52±0.06 for pattern B LDL and 2.66±0.43 for pattern A LDL.
The release of NO/ONOO⁻ is dose-dependent, which is proportional to the concentration of LDL directly injected to the endothelial cells. NO production was increased from 33±4 nmol/L, 40±5 nmol/L and 74±10 nmol/L (at 50 µg/mL of LDL injection, control) to 211±18 nmol/L, 300±28 nmol/L and 591±73 nmol/L for LDL of pattern B, I and A (at 1000 µg/mL of LDL injection), respectively (Figure 3.7). ONOO⁻ production was increased from 103±2 nmol/L, 80±4 nmol/L and 54±6 nmol/L (at 50 µg/mL of LDL injection) to 409±36 nmol/L, 323±29 nmol/L, and 222±23 nmol/L for LDL of pattern B, I and A (at 1000 µg/mL of LDL injection), respectively (Figure 3.8). In addition, the ratio of [NO] to [ONOO⁻] maintained in the range from 0.29 to 0.52 for pattern B LDL, which indicates that endothelial cells were under high oxidative stress.
For pattern I and A LDL injection, the ratios changed from 0.50 to 0.93 and from 1.37 to 2.66, respectively (Figure 3.9).

Figure 3.7 Dose-dependent NO release from the surface of endothelial cells after direct injection of LDL with different patterns (A, B and I) and concentrations (from 50 µg/mL to 1000 µg/mL). Data are expressed as mean±SD.
Figure 3.8 Dose-dependent ONOO⁻ release from the surface of endothelial cells after direct injection of LDL with different patterns (A, B and I) and concentrations (from 50 µg/mL to 1000 µg/mL). Data are expressed as mean±SD.
Figure 3.9 The ratio of [NO] to [ONOO·] measured in endothelial cells after direct injection of LDL with different patterns (A, B and I) and concentrations (from 50 µg/mL to 1000 µg/mL). Data are expressed as mean±SD.

We also investigated the effect of LDL with different mixture combinations on endothelial cells. The experiment was carried out by directly injecting LDL in seven of different mixture combinations to the surface of endothelial cells: (1) 60% A, 20% B and 20% I; (2) 20% A, 60% B and 20% I; (3) 20% A, 20% B and 60% I; (4) 50% A and 50% B; (5) 50% A and 50% I; (6) 50% B and 50% I; (7) 33% A, 38% B and 29% I (simulation of original constituent from general human plasma). Our data showed that the first group (60% A, 20% B and 20% I) stimulated endothelial cells to release the lowest concentration of ONOO· (77±8 nmol/L) and highest concentration of NO (436±28
nmol/L), while the last group (50% B and 50% I) stimulated endothelial cells to release the highest concentration of ONOO\(^-\) (369±25 nmol/L) and lowest concentration of NO (166±10 nmol/L). The ratio of [NO] to [ONOO\(^-\)] concentration was 5.66±0.69 for the first group, and 0.45±0.04 for the last group (Figure 3.10, 3.11).

Figure 3.10 [NO] and [ONOO\(^-\)] release from endothelial cells stimulated by LDL injection with different mixture combinations (800 µg/mL). Close bar indicates NO and open bar indicates ONOO\(^-\). Data are expressed as mean±SD.
Furthermore, the effect of multiple agents on endothelial cells combined with LDL injection was investigated as well. Specifically, all reagents except L-NAME increased NO production after LDL injection with pattern A, B and I. With pattern A LDL injection, NO concentrations were 129%, 159%, 231%, 41% and 124% of control for treatment of PEG-SOD, L-arginine, sepiapterin, L-NAME and VAS2870, respectively. Pattern I LDL injection stimulated NO release at 120%, 141%, 161%, 54% and 121% of control for treatment of PEG-SOD, L-arginine, sepiapterin, L-NAME and VAS2870, respectively. And for pattern B LDL injection, NO concentrations were 125%, 142%, 165%, 67% and 115% of control for treatment of PEG-SOD, L-arginine, sepiapterin, L-NAME and VAS2870, respectively (Figure 3.12).
Figure 3.12 NO release from endothelial cells stimulated by LDL injection (Pattern A, I and B, 800 µg/mL) after incubation with different treatments. Endothelial cells were pre-treated with control (EBM), PEG-SOD (400 U/mL), L-arginine (300 µM), sepiapterin (200 µM), L-NAME (100 µM) and VAS2870 (10 µM) at 37 ºC for 30 minutes. Data are expressed as mean±SD.

ONOO⁻ production was decreased for all supplementations after LDL injection with pattern A, B and I. With pattern A LDL injection, ONOO⁻ concentrations were 55%, 57%, 54%, 61% and 73% of control for treatment of PEG-SOD, L-arginine, sepiapterin, L-NAME and VAS2870, respectively. Pattern I LDL injection stimulated ONOO⁻ release at 64%, 59%, 74%, 54% and 76% of control for treatment of PEG-SOD, L-arginine,
sepiapterin, L-NAME and VAS2870, respectively. And for pattern B LDL injection, ONOO- concentrations were 59%, 52%, 73%, 55% and 80% of control for treatment of PEG-SOD, L-arginine, sepiapterin, L-NAME and VAS2870, respectively (Figure 3.13).

Figure 3.13 ONOO$^-$ release from endothelial cells stimulated by LDL injection (Pattern A, I and B, 800 µg/mL) after incubation with different treatments. Endothelial cells were pre-treated with control (EBM), PEG-SOD (400 U/mL), L-arginine (300 µM), sepiapterin (200 µM), L-NAME (100 µM) and VAS2870 (10 µM) at 37 °C for 30 minutes. Data are expressed as mean±SD.
With pattern A LDL injection, the ratio of NO to ONOO^− concentration was 1.90, 4.49, 5.31, 8.18, 1.28 and 3.20 for treatment of control, PEG-SOD, L-arginine, sepiapterin, L-NAME and VAS2870, respectively. For pattern I LDL injection, the ratio was 0.84, 1.58, 2.01, 1.82, 0.83 and 1.33 for treatment of control, PEG-SOD, L-arginine, sepiapterin, L-NAME and VAS2870, respectively. And for pattern B LDL injection, the ratio was 0.42, 0.88, 1.15, 0.94, 0.50 and 0.60 for treatment of control, PEG-SOD, L-arginine, sepiapterin, L-NAME and VAS2870, respectively (Figure 3.14).

Figure 3.14 Ratio of [NO] to [ONOO^−]. Data are expressed as mean±SD.
3.2.2 Ox-LDL induced NO and ONOO⁻ release in endothelial cells

In this study, we also investigated the effect of n-LDL and ox-LDL with different patterns on endothelial cells. Ox-LDL injection can stimulate endothelial cells to release less NO than n-LDL, 267±11 nmol/L vs 418±16 nmol/L for pattern A LDL, 95±7 nmol/L vs 152±10 nmol/L for pattern I LDL, and 65±3 nmol/L vs 85±3 nmol/L for pattern B LDL (Figure 3.15). Meanwhile, ox-LDL induced more ONOO⁻ production than n-LDL, 145±6 nmol/L vs 86±5 nmol/L for pattern A LDL, 284±18 nmol/L vs 208±13 nmol/L for pattern I LDL, and 432±18 nmol/L vs 347±20 nmol/L for pattern B LDL (Figure 3.16). And the ratio of [NO] to [ONOO⁻] is 1.84 (ox-LDL) vs 4.86 (n-LDL), 0.33 vs 0.73 and 0.15 vs 0.24 for pattern A, B and I LDL, respectively (Figure 3.17).

Figure 3.15 NO production induced by ox-LDL/n-LDL (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs n-LDL (n=4).
Figure 3.16 ONOO⁻ production induced by ox-LDL/n-LDL (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs n-LDL (n=4).

Figure 3.17 Ratio of [NO] to [ONOO⁻]. Data are expressed as mean±SD.
3.2.3 n-LDL/ox-LDL induced NO and ONOO⁻ release in endothelial cells of African Americans (AA) and Caucasian Americans (CA)

NO released from endothelial cells of CA was lower than AA for stimulation with n-LDL subpattern A and I (325±3 vs 411±16 nmol/L, and 191±11 vs 249±18 nmol/L, \( p<0.01 \)), while pattern B induced NO release from endothelial cells of CA was close to AA (87±5 vs 96±8 nmol/L, \( p<0.05 \)) (Figure 3.18). ONOO⁻ release from endothelial cells of CA was also lower than AA for stimulation with n-LDL subpattern A, I, and B (111±8 vs 155±5 nmol/L, 165±5 vs 230±12 nmol/L, and 340±10 vs 407±12 nmol/L, \( p<0.01 \)) (Figure 3.19). However, the ratio of [NO] to [ONOO⁻] was lower in endothelial cells of AA than in CA (Figure 3.20).

Figure 3.18 Differences in peak NO release from HUVECs of African Americans (solid bars) and Caucasian Americans (open bars). Stimulation with n-LDL of pattern A, I and B (800 µg/mL). Data are expressed as mean±SD. *\( P<0.01 \) vs African Americans (n=4).
Figure 3.19 Differences in peak ONOO⁻ release from HUVECs of African Americans (solid bars) and Caucasian Americans (open bars). Stimulation with n-LDL of pattern A, I and B (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4).
Figure 3.20 Ratio of [NO] to [ONOO\textsuperscript{-}]. HUVECs of African Americans (solid bars) and Caucasian Americans (open bars). Data are expressed as mean±SD.

To investigate the effect of ox-LDL stimulation in both racial groups, endothelial cells of AA and CA were stimulated by ox-LDL of pattern A, B and I, the release of NO and ONOO\textsuperscript{-} were measured in the same way as described previously. The result of ox-LDL stimulated NO and ONOO\textsuperscript{-} release is similar to stimulation with n-LDL. In the CA group, NO release was lower than the AA group (163±12 vs 229±15 nmol/L, 61±3 vs 92±4 nmol/L, and 42±3 vs 55±4 nmol/L for pattern A, I and B, respectively; p<0.01 vs AA). ONOO\textsuperscript{-} release from endothelial cells of CA also showed lower concentration than AA (169±9 vs 217±6 nmol/L, 248±10 vs 429±14 nmol/L, and 377±23 vs 602±36 nmol/L for pattern A, I and B, respectively; p<0.01 vs AA), suggesting that HUVECs of AA are more susceptible to ox-LDL stimulation than endothelial cells of CA (Figure 3.21, 3.22).
However, the ratio of [NO] to [ONOO⁻] is very close between CA and AA, 0.96 vs 1.06 for pattern A, 0.25 vs 0.21 for pattern I, and 0.11 vs 0.09 for pattern B (Figure 3.23).

Figure 3.21 Maximal NO released from endothelial cells of African Americans (solid bars) and Caucasian Americans (open bars). Stimulation with ox-LDL of pattern A, B and I (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4).
Figure 3.22 Maximal ONOO$^-$ released from endothelial cells of African Americans (solid bars) and Caucasian Americans (open bars). Stimulation with ox-LDL of pattern A, B and I (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4).
We also investigated the effect of n-LDL with different mixture combinations on endothelial cells of CA and AA. The experiment was carried out by directly injecting LDL of seven different mixture combinations to the surface of endothelial cells: (1) 60% A, 20% B and 20% I; (2) 20% A, 60% B and 20% I; (3) 20% A, 20% B and 60% I; (4) 50% A and 50% B; (5) 50% A and 50% I; (6) 50% B and 50% I; (7) 33% A, 38% B and 29% I (simulation of original constituent from general human plasma). Our data showed that the first group (60% A, 20% B and 20% I) stimulated endothelial cells to release the highest concentration of NO (362±13 nmol/L for AA and 284±14 nmol/L for CA) and lowest concentration of ONOO⁻ (105±6 nmol/L for AA and 83±4 nmol/L for CA), while the last group (50% B and 50% I) stimulated endothelial cells to release the lowest concentration of NO (99±5 nmol/L for AA and 76±6 nmol/L for CA) and highest
concentration of ONOO$^-$ (365±6 nmol/L for AA and 309±6 nmol/L for CA). When injected with the same combination of n-LDL, the AA group showed higher concentration of NO release than CA (NO increased up to 127%, 131%, 140%, 118%, 156%, 130% and 119% of CA for group 1 to group 7, respectively), while ONOO$^-$ release was higher in AA than CA as well (ONOO$^-$ increased up to 127%, 117%, 127%, 120%, 110%, 118% and 107% of CA for group 1 to group 7, respectively). The ratio of [NO] to [ONOO$^-$] in AA is close to that in CA among different groups, except for group 5 (50% A and 50% I), which is 2.11±0.48 in AA vs 1.48±0.39 in CA (Figure 3.24-3.26).

Figure 3.24 NO release from HUVECs of African Americans (solid bars) and Caucasian Americans (open bars) after stimulation with n-LDL of different mixture combinations (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4).
Figure 3.25 ONOO\textsuperscript{-} release from HUVECs of African Americans (solid bars) and Caucasian Americans (open bars) after stimulation with n-LDL of different mixture combinations (800 µg/mL). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=4).
Figure 3.26 Ratio of [NO] to [ONOO⁻]. African Americans (solid bars) and Caucasian Americans (open bars). Data are expressed as mean±SD.

3.3 Effects of LDL on ICAM-1 and VCAM-1 expression in HUVECs

3.3.1 Effects of n-LDL on ICAM-1 and VCAM-1 expression in HUVECs

To determine the effect of different subpatterns of n-LDL on ICAM-1 and VCAM-1 expression, endothelial cells were incubated with basal medium containing 400 µg/mL n-LDL/ox-LDL (pattern A, B and I) for 5 hours and the expression of ICAM-1 and VCAM-1 was measured by cell ELISA. ICAM-1 expression significantly increased in cells stimulated by LDL, 155±11%, 174±14% and 190±7% of control for n-LDL of pattern A, I, and B stimulation, respectively. VCAM-1 expression was also increased after n-LDL stimulation, 160±5%, 178±4% and 209±10% of control for pattern A, I and B stimulation, respectively (Figure 3.27).
Figure 3.27 Effect of n-LDL incubation with different patterns on the expression of ICAM-1 (solid bars) and VCAM-1 (open bars). Data are expressed as mean±SD. OD$_{450}$ indicates optical density at 450 nm wavelength. *P<0.01 vs control (n=6).

3.3.2 Effects of ox-LDL on ICAM-1 and VCAM-1 expression in HUVECs

Ox-LDL increased ICAM-1 expression by about 150%, 170% and 190% of control for pattern A, I and B, respectively. This is about 10% ~ 20% more than ICAM-1 expression stimulated by n-LDL (Figure 3.28). VCAM-1 expression induced by ox-LDL was about 120%, 150% and 190% of control for pattern A, I and B, respectively. This is about 10% ~ 40% more than VCAM-1 expression stimulated by n-LDL (Figure 3.29).
Figure 3.28 Effect of different subpatterns of ox-LDL/n-LDL on the expression of ICAM-1. Data are expressed as mean±SD. OD_{450} indicates optical density at 450 nm wavelength. *P<0.01 vs control (n=6).
Figure 3.29 Effect of different subpatterns of ox-LDL/n-LDL on the expression of VCAM-1. Data are expressed as mean±SD. OD$_{450}$ indicates optical density at 450 nm wavelength. *P<0.01 vs control (n=6).

3.3.3 Effects of n-LDL on ICAM-1 and VCAM-1 expression in HUVECs from AA and CA

Endothelial cells were incubated in basal medium containing 400 μg/mL n-LDL/ox-LDL (pattern A, B and I) for 5 hours and the expression of ICAM-1 and VCAM-1 was measured by cell ELISA. ICAM-1 expression increased significantly in endothelial cells of AA, about 20% – 25% more than CA (Figure 3.30). VCAM-1 expression was also increased, about 20% – 40% more than CA (Figure 3.31).
Figure 3.30 ICAM-1 expression stimulated by n-LDL of pattern A, I and B (400 µg/mL). 

OD$_{450}$ indicates optical density at 450 nm wavelength. African Americans (solid bars) and Caucasian Americans (CA). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6).
Figure 3.31 VCAM-1 expression induced by n-LDL of pattern A, I and B (400 µg/mL). OD\textsubscript{450} indicates optical density at 450 nm wavelength. African Americans (solid bars) and Caucasian Americans (CA). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6).

Ox-LDL stimulated ICAM-1 expression in HUVECs of AA was higher than expression in CA; 143%, 129% and 121% of CA for pattern A, I and B, respectively (Figure 3.32). In addition, VCAM-1 expression induced by ox-LDL was 143%, 136% and 119% of control for pattern A, I and B, which showed 6%, 23% and 42% more than ICAM-1 expression stimulated by n-LDL with pattern A, I, and B, respectively (Figure 3.33).
Figure 3.32 ICAM-1 expression induced by ox-LDL of pattern A, I and B (400 µg/mL). OD$_{450}$ indicates optical density at 450 nm wavelength. African Americans (solid bars) and Caucasian Americans (CA). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6).
Figure 3.33 VCAM-1 expression induced by ox-LDL of pattern A, I and B (400 µg/mL). 
OD_{450} indicates optical density at 450 nm wavelength. African Americans (solid bars) and Caucasian Americans (CA). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6).

3.4 LDL induced cell adhesion in endothelial cells

3.4.1 n-LDL induced cell adhesion in endothelial cells

To investigate monocyte adhesion to endothelial cells in the presence of LDL, we measured the fluorescence intensity of pre-labeled THP-1 cells. Our data showed that, after 60 minutes incubation, pattern B LDL induced the highest level of monocyte adhesion to endothelial monolayer, while pattern A LDL induced the lowest level of monocyte adhesion. Pattern I LDL induced monocyte adhesion was somewhere in between (Figure 3.34).
The effect of LDL concentration on monocytes adhesion was also investigated. THP-1 cells adhesion is dose-dependent, 400 µg/mL LDL treatment induced the maximal monocytes adhesion while 50 µg/mL LDL treatment induced the minimal adhesion. At the same concentration, LDL of different patterns induced monocytes adhesion differently, pattern B LDL induced the highest level of monocyte adhesion while pattern A stimulated the lowest level of monocyte adhesion (Figure 3.35).
Figure 3.35 Dose-dependent monocyte adhesion induced by incubation with n-LDL of pattern A, I and B (50, 100, 200, 400 µg/mL). Data are expressed as mean±SD. MFI indicates mean fluorescence intensity.

3.4.2 Ox-LDL induced cell adhesion in endothelial cells

Ox-LDL treatment group induced more monocytes adhesion than n-LDL group: pattern A, I and B increased monocyte adhesion about 20%, 70% and 60% more than n-LDL, respectively. In ox-LDL treatment group, pattern B induced the highest level of monocyte adhesion (370% of control), while pattern A induced the lowest level of monocyte adhesion (187% of control), and pattern I induced intermediate level of monocyte adhesion (286% of control). This is similar to n-LDL incubation (Figure 3.36).
Figure 3.36 Monocyte adhesion induced by n-LDL/ox-LDL of pattern A, I and B (400 µg/mL). Data are expressed as mean±SD. MFI indicates mean fluorescence intensity. *P<0.01 vs n-LDL (n=6).

### 3.4.3 n-LDL and ox-LDL induced cell adhesion in HUVECS of African Americans and Caucasian Americans

We measured the fluorescence intensity of pre-labeled THP-1 cells adhered to endothelial monolayer. n-LDL induced monocyte adhesion was higher in endothelial cells of AA than that in CA endothelial cells for all patterns of n-LDL. Monocyte adhesion in endothelial cells of AA was about 20% ~ 60% more than in endothelial cells of CA (Figure 3.37). In addition, the effect of ox-LDL on monocytes adhesion was investigated. Monocyte adhesion in endothelial cells of AA was about 25% ~ 40% higher
than endothelial cells of CA, suggesting that ox-LDL induced monocyte adhesion is race-specific as well (Figure 3.38).

Figure 3.37 Race-specific monocyte adhesion stimulated by different n-LDL subpatterns (400 μg/mL). MFI indicates mean fluorescence intensity. African Americans (solid bars) and Caucasian Americans (open bars). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6).
Figure 3.38 Race-specific monocyte adhesion stimulated by different ox-LDL subpatterns (400 µg/mL). MFI indicates mean fluorescence intensity. African Americans (solid bars) and Caucasian Americans (open bars). Data are expressed as mean±SD. *P<0.01 vs African Americans (n=6).
4 Discussion

4.1 Separation and oxidation of LDL subpatterns

In this study, we confirmed heterogeneity of LDL. Three major subpatterns of LDL with different density were successfully separated from pooled human plasma through ultracentrifugation: pattern A (1.016-1.019 g/mL), pattern I (1.024-1.029 g/mL), and pattern B (1.034-1.053 g/mL). Our results show that this novel separation method has good repeatability (Figure 3.1) and thus it can ensure the uniformity of LDL subpatterns. Iodixanol was applied as a new non-ionic, iodinated-density gradient medium, which can self-generate a density gradient during ultracentrifugation.\textsuperscript{76, 77, 136} The conventional way to separate LDL subfractions is carried out by ultracentrifugation with a discontinuous or continuous gradient of potassium bromide, which is time-consuming (up to 78 hours for sequential flotation or at least 24 hours for separation on discontinuous gradients) and requires overnight-dialysis to remove salt.\textsuperscript{75} This new method is simple and fast: plasma is mixed with iodixanol followed by four hours ultracentrifugation.

LDL subpatterns separated by this new method are comparable in density and composition with those separated in salt-based gradients. However, these subpatterns of LDL are slightly less dense than those in salt gradients. Because the iodixanol-gradients are essentially iso-osmotic, keeping protein molecules with their native hydration status, rather than loss of water in highly hyper-osmotic salt gradients, which causes the increase in density. Iodixanol has low cytotoxicity and can help LDL samples maintain their native hydration status, thus there is no need to use overnight-dialysis to remove iodixanol.
LDL cholesterol has been considered as “bad” cholesterol while HDL is considered as “good” cholesterol. However, this classification is arbitrary. In fact, all forms of cholesterol are essential for life and therefore are good. The main reason why LDL is called “bad” cholesterol is that LDL may form a plaque in arteries, slow down blood flow, weaken artery wall, cause an inflammation, develop atherosclerosis, and eventually increase the risk of heart disease.

However, in the recent decades, more and more studies have been reported, which suggest that it is not an accurate description. LDL becomes a bad cholesterol occasionally. LDL is not homogeneous and only small and dense LDL (sdLDL or pattern B LDL) has a strong correlation with high risk of CVD and can be called “bad” cholesterol. The heterogeneity of LDL was first found by Lindgren and colleagues and then confirmed by other groups. Based on distinct density and particle size, LDL is classified into several subpatterns, including pattern A, which contains more of the larger and less dense LDL particles with density of 1.025–1.034 g/mL; pattern B, which has more of the smaller and more dense LDL particles with density of 1.044–1.060 g/mL; and pattern I has medium size particles with density of 1.034-1.044 g/mL. It has been shown that pattern B LDL is strongly associated with increased cardiovascular risk. However, the effect of different LDL subpatterns in the cardiovascular system, especially in endothelial cells, has not been fully studied yet. In this work, we investigated the effect of different LDL subpatterns on stimulating the release of NO and ONOO⁻ from endothelial cells, the expression of ICAM-1 and VCAM-1 as well as monocyte adhesion.
Previous studies have already reported the variations in oxidative susceptibility of LDL subpatterns with different density.\textsuperscript{85, 86} Lag time is the parameter that is used to evaluate the susceptibility of LDL subpatterns to oxidation. Longer lag time indicated less susceptibility to oxidative modifications. Several studies have reported that pattern B LDL is more susceptible to oxidation than pattern A LDL based on lag time for completing oxidation of LDL particles.\textsuperscript{83, 87} Once native LDL turns to oxidized-LDL, which becomes more cytotoxic because of reduction NO production,\textsuperscript{88} increasing oxidative stress, up-regulating the expression of adhesion molecules\textsuperscript{142, 143} and stimulating monocyte adhesion,\textsuperscript{144} finally causing the premature development of atherosclerosis.

4.2 n-LDL/ox-LDL induced an imbalance of NO and ONOO$^-$ in endothelial cells

In this study, we investigated the role of different LDL subpatterns in induction of nitroxidative stress in endothelial cells. Different subpatterns of LDL stimulated NO and ONOO$^-$ release differently for both n-LDL and ox-LDL. Our data suggest that pattern B LDL can stimulate endothelial cells to produce the highest level of ONOO$^-$ and the lowest level of NO production, resulting in imbalanced [NO] to [ONOO$^-$] ratio, causing endothelial dysfunction and finally aggravating nitroxidative stress in endothelial cells.

4.2.1 n-LDL stimulated NO and ONOO$^-$ release in endothelial cells

Our data show that the release of NO and ONOO$^-$ is positive correlated with the concentration of LDL subpatterns, suggesting the stimulation effect of LDL subpatterns is dose-dependent (Figure 3.7 and 3.8). However, the maximal NO/ONOO$^-$ release from endothelial cells was different among LDL subpatterns. NO release stimulated by pattern
A LDL is higher than pattern B LDL. NO release stimulated by pattern I LDL is intermediate. In contrast, the maximal release of ONOO$^-$ stimulated by pattern B LDL is higher than pattern A LDL, and pattern I LDL stimulation is still intermediate.

The ratio of [NO] to [ONOO$^-$] indicates the oxidative/nitroxidative stress level and NO/ONOO$^-$ balance in endothelial cells. High ratio (>2.0) indicates high level of NO bioavailability and/or relative low concentration of cytotoxic ONOO$^-$. A ratio below 1.0 suggests that the cellular environment is dominated by high oxidative/nitroxidative stress and endothelium is dysfunctional. It was 0.5 for pattern B, suggesting that endothelial cells were dysfunctional; and it was 2.7 for pattern A and 0.9 for pattern I, indicating that endothelial cells were functional or at least close to functional status (Figure 3.6).

Under normal conditions, cNOS can synthesize NO from L-arginine; while under dysfunctional status, cNOS will be uncoupled and catalyze the reaction between NO and O$_2^-$ to form ONOO$^-$.\textsuperscript{92,110} LDL can induce a rapid release of NO and superoxide (O$_2^-$) in cultured endothelial cells.\textsuperscript{88} Our data reveal that pattern B LDL can cause severe dysfunction in endothelial cells and imbalance of NO/ONOO$^-$ ratio by decreasing the bioavailability of NO and increasing ONOO$^-$ production, which is the main component of nitroxidative stress. Meanwhile, pattern A LDL can keep endothelial cells functional and maintain the normal ratio of NO/ONOO$^-$. Therefore, the density difference in LDL particles can be used as a diagnostic predictor in estimating the level of endothelial dysfunction and oxidative/nitroxidative stress.

We also tried different combinations of LDL subpatterns to simulate the possible composition of LDL in plasma. Our data suggested that mixture of 50% B and 50% I
stimulated endothelial cells to release the highest concentration of ONOO$^-$ (369±25 nmol/L), while mixture of 60% A, 20% B and 20% I significantly decreased ONOO$^-$ production (77±8 nmol/L). But for NO production, the result was different. Mixture of 60% A, 20% B and 20% I stimulated the highest concentration of NO (436±28 nmol/L) while mixture of 50% B and 50% I induced the lowest concentration of NO release (166±10 nmol/L) (Figure 3.10). In addition, the ratio of [NO] to [ONOO$^-$] for mixture of 50% B and 50% I LDL was the lowest, and the ratio for mixture of 60% A, 20% B and 20% I was the highest (Figure 3.11), suggesting that the former caused severe imbalance of NO and ONOO$^-$ while the latter kept endothelial cells in normal functional status.

The original distribution of different LDL subpatterns in the general LDL sample isolated from pooled human plasma is 33% A, 38% B and 29% I. We also investigated its effect on endothelial cells. The ratio of [NO] to [ONOO$^-$] was about 0.8, indicating that general LDL can cause slight endothelial dysfunction as well as imbalance of NO and ONOO$^-$, but this effect was not as severe as the mixture of 50% B and 50% I LDL. Those data suggested that the composition of LDL subpatterns is detrimental in the induction of nitrooxidative stress: high percentage of pattern B stimulated high level of ONOO$^-$ with low level of NO; while high percentage of pattern A stimulated more NO than ONOO$^-$.

Therefore, analyzing the distribution of LDL subpatterns may provide a parameter-based model for more precise diagnosis of estimating risk of cardiovascular disease.

Furthermore, we investigated the effect of multiple NO and ONOO$^-$ modulators on endothelial cells. In each LDL subpattern stimulated group, NO release was higher than control for all treatments except for L-NAME; and ONOO$^-$ release was lower than
control for all treatments. In PEG-SOD treatment group, NO stimulated by LDL subpatterns was higher than control group; meanwhile, ONOO\(^-\) production was lower than control group, suggesting that PEG-SOD has endogenous antioxidant capacity by increasing NO bioavailability and reducing ONOO\(^-\) production. As a free-radical scavenger, SOD can catalyze the dismutation of superoxide (O\(_2^-\)) into oxygen and hydrogen peroxide, resulting in a decrease of ONOO\(^-\) production. Meanwhile, SOD can increase NO generation by enhancing its stability. However, native SOD has a very short half-life (5-10 minutes) and is incapable of penetrating across cell membranes, which makes it difficult to apply in many experiments. Thus SOD is covalently linked to polyethylene glycol (PEG-SOD) to extend its short half-life and gain access into intracellular space; thereby the enhancement of endogenous antioxidant capacity of PEG-SOD can facilitate its application to *in vivo* or *in vitro* experiments.\(^{145}\) Previous studies have reported the extended half-life of PEG-SOD in dogs’ plasma beyond 30 hours.\(^{146, 147}\) One study reported that there was a bell-shaped dose-response for the protective properties of PEG-SOD, suggesting that either excessive or insufficient concentration of PEG-SOD may lead to some unexpected or negative results.\(^{148}\) We used 400U/mL of PEG-SOD for incubation in this study. However, it may be worth carrying out the same experiment under different concentrations of PEG-SOD and the results attributed to the interaction of PEG-SOD and LDL subpatterns may further reveal the cytoprotective and antioxidant capacity of PEG-SOD in physiological surroundings.

In the L-arginine treatment group, NO production increased significantly for all of LDL subpatterns compared to control group. Meanwhile, ONOO\(^-\) production reduced
dramatically. NO is biosynthesized from L-arginine by eNOS, and thereby, as the substrate for NO production, increasing the supplementation of L-arginine can restore the normal status of eNOS and balance of [NO] to [ONOO⁻] ratio by enhancing NO production. Vergnani and coworkers reported that L-arginine treatment with endothelial cells before LDL incubation can increase NO production and decrease ONOO⁻ generation. One possible reason may be that sufficient supplementation of L-arginine coupled with eNOS can restore normal activity of eNOS and bioavailability of NO, therefore the synthesis pathway of ONOO⁻ is turned down at the presence of L-arginine, leading to the reduction of ONOO⁻ production.

In the sepiapterin treatment group, NO production was also higher than control for all of LDL subpatterns. Meanwhile, ONOO⁻ production reduced dramatically compared to control. Sepiapterin is a precursor of eNOS cofactor tetrahydrobiopterin (BH₄), which can convert to BH₄ via a salvage pathway by sepiapterin reductase and dihydrofolate reductase, thereby helping endothelial NOS maintain functional status with catalytic activity and normal balance of [NO] and [ONOO⁻] by increasing NO biosynthesis from L-arginine. Our data suggest that uncoupling of NOS induced by LDL subpatterns can be reversed by supplementation of sepiapterin. However, pattern A LDL still stimulated the highest level of NO and the lowest level of ONOO⁻, and on the contrary, pattern B LDL stimulated the lowest level of NO and the highest level of ONOO⁻, suggesting that pattern B LDL can cause more severe endothelium dysfunction than pattern A LDL.
In the L-NAME treatment group, both NO and ONOO$^-$ production reduced significantly. As an L-arginine analogue and nonspecific inhibitor of cNOS, L-NAME can bind to the active site of cNOS to block its catalytic activity, resulting in reducing the production of both NO and ONOO$^-$.\textsuperscript{150} However, this substrate analogue-mediated inhibition of NOS activity is reversible with sufficient supplementation of L-arginine.\textsuperscript{151} Compared to its unesterified form L-nitro-arginine (L-NA), L-NAME is more soluble in water than L-NA due to esterification of the carboxyl group, which facilitates the experimental application of L-NAME, therefore it is widely used to inhibit NO production in both \textit{in vivo} and \textit{in vitro} studies. It is very interesting that some studies reported increased NOS activity in low dose treatment of L-NAME, which may upregulate NO production via feedback regulatory mechanisms as well as increase the expression level of NOS.\textsuperscript{152-154} However, it does not mean that higher bioavailability of NO is necessarily in association with increased NOS expression and/or NOS activity. Since NO biosynthesis is determined by many factors, for instance, lack of cofactors needed for NOS activation, oxidation and/or inactivation of BH$_4$ and the presence of highly reactive ROS can reduce NO production.\textsuperscript{41, 155} Nevertheless, it is also worthwhile to investigate the dosage-effect of L-NAME on NO and ONOO$^-$ release from endothelial cells in association with different LDL subpatterns.

In the VAS2870 treatment group, NO production increased significantly while ONOO$^-$ production decreased dramatically, compared to control group. As the major product of NADPH oxidases, and one of reactive oxygen species (ROS), O$_2^-$ can oxidize NO to form ONOO$^-$, which contributes to oxidative stress in endothelium and leads to
endothelial dysfunction. VAS2870 can permeate cell membrane and inhibit NADPH oxidase activity in a rapid and reversible way. VAS2870 can also repeal agonist-induced ROS production and thereby provides protection against oxidative stress generated by ROS. In this study, NO concentration was increased by 15-24% of control group, suggesting that this portion of NO produced by endothelial cells is consumed by O$_2^-$ generated by NADPH oxidase to form ONOO$^-$. Meanwhile, ONOO$^-$ concentration was decreased by 20-27% of control group, which was consistent with the increase of NO production (Figure 3.12 and 3.13).

It is interesting that both NO and ONOO$^-$ release in each treatment group showed the similar trends as control group: pattern B stimulated the lowest level of NO and the highest level of ONOO$^-$, while pattern A stimulated the highest level of NO and the lowest level of ONOO$^-$, suggesting that the distinct production of NO and ONOO$^-$ stimulated by LDL subpatterns is insusceptible to those NO and ONOO$^-$ modulators used in this study. The only exception is the L-NAME treatment group, which showed equivalent concentration of NO stimulated by all of LDL subpatterns, suggesting that the inhibition of NOS activity by high-dose level (100 µmol/L) of L-NAME is so strong that it suppressed NO production induced by LDL to the same level and thus diminished the different effect among LDL subpatterns.

4.2.2 Ox-LDL induced NO and ONOO$^-$ release from endothelial cells

In the present study, we compared the effect of ox-LDL and n-LDL with different subpatterns on stimulating NO and ONOO$^-$ release from endothelial cells. Ox-LDL reduced NO release and increased ONOO$^-$ release compared to n-LDL stimulation,
suggesting that ox-LDL can cause more severe endothelium dysfunction and higher level of nitrooxidative stress than n-LDL. Our data also suggested that pattern A LDL was affected the most through oxidative modification, as it reduced the highest percentage of NO production and increased the highest percentage of ONOO\(^-\) release compared to pattern I and B. However, stimulation with ox-LDL of pattern A still kept endothelial cells under functional status, as the ratio was about 2.0. Compared to n-LDL, ox-LDL showed even lower ratio for the same LDL subpatterns. The ratio decreased about 60% on average of all subpatterns (Figure 3.17), suggesting that LDL became more cytotoxic and cause even worse endothelial dysfunction after oxidative modification.

LDL oxidation plays a fundamental role in the formation and progression of early atherosclerotic lesions. Ox-LDL can bind to scavenger receptor, leading to the accumulation of cholesteryl esters in macrophages and induction of cytotoxicity in endothelial cells. Among different LDL subpatterns, pattern B is the most susceptible to oxidation.\(^{81}\) Incubation with ox-LDL/n-LDL can stimulate ONOO\(^-\) release and inhibit NO production from endothelial cells.\(^{88}\) In this study, our data show for the first time that, ox-LDL stimulated less NO production and more ONOO\(^-\) release than n-LDL, suggesting that ox-LDL is more cytotoxic than n-LDL in induction of endothelial dysfunction and imbalance of NO to ONOO\(^-\) ratio, which may play an important role in the pathogenesis of atherosclerosis.
4.2.3 n-LDL/ox-LDL induced NO and ONOO⁻ release in endothelial cells from African Americans and Caucasian Americans

In this study, NO released from HUVECs of AA was higher than CA, suggesting that HUVECs of AA are more susceptible to LDL stimulation than HUVECs of CA. However, the differences between AA and CA were more significant in pattern A than pattern B, indicating that the race-specific effect of LDL is distinct among different LDL subpatterns. ONOO⁻ release was also higher in AA than that in CA, which was similar to NO release. But for endothelial cells from the same race, ONOO⁻ release showed the same pattern with pooled endothelial cells in our previous findings, suggesting that the different effects of LDL subpatterns on NO and ONOO⁻ release were determined by LDL subpatterns rather than the source of endothelial cells.

The [NO] to [ONOO⁻] ratio in endothelial cells of AA was lower than CA, indicating that the steady balance of NO and ONOO⁻ in endothelial cells of AA were kept closer to the redox state than CA. For pattern I and B LDL, the ratio difference between AA and CA was not as significant as pattern A LDL, suggesting that the effect of small and dense LDL on inducing endothelial dysfunction is so strong that it can diminish the race-specific differences in response to LDL stimulation. Similar results were obtained with ox-LDL stimulation: both NO and ONOO⁻ release was higher in AA group than that in CA, suggesting that HUVECs of AA are more susceptible to ox-LDL stimulation than CA. NO to ONOO⁻ ratio was lower in endothelial cells of AA than CA, suggesting that the oxidative stress caused by ox-LDL stimulation is higher in AA than in CA. For pattern I and B LDL, the ratio difference between AA and CA is similar as n-LDL
stimulation, suggesting that LDL subpatterns are determinants in the induction of endothelial dysfunction and nitrooxidative stress regardless of race.

We also investigated the effect of n-LDL with different combinations on HUVECs of AA and CA. Both NO and ONOO\(^{-}\) release was higher in AA than CA, and the [NO] to [ONOO\(^{-}\)] ratio was similar between AA and CA for most combinations, except for group 3 (20% A, 20% B and 60% I), group 5 (50% A and 50% I) and group 7 (33% A, 38% B and 29% I), among which the ratio was higher in AA than that in CA (Figure 3.26).

It’s interesting that LDL stimulation with different combinations has distinct effect on HUVECs compared to LDL stimulation with single subpattern. When mixed together, LDL subpatterns may interact with each other, generating a mixed-effect which is more complicated than single pattern effect, thus the result is different from stimulation with single pattern LDL. Previous study reported that HUVECs of AA released lower bioavailable NO and higher ONOO\(^{-}\) than CA after stimulation with 1.0 µmol/L calcium ionophore A23187 (CaI).\(^{120}\) CaI is an eNOS agonist that can stimulate NO production from endothelial cells by activating the catalytic function of eNOS. NO and ONOO\(^{-}\) release stimulated by LDL subpatterns may be through different mechanisms, which may lead to the different result between endothelial cells of AA and CA.

Accumulating evidence has shown that Blacks experience a higher cardiovascular morbidity and mortality compared to Whites.\(^{1,117,158}\) However, the reason why Blacks are at greater risk of developing cardiovascular diseases than Whites remains unclear. One possible explanation proposed that, compared to endothelial cells of CA, the reduced
bioavailability of NO and increased nitoxidative stress in the endothelial cells of AA may lead to endothelium-impaired dysfunction, which is responsible for the higher rates of mortality in cardiovascular complications. In summary, we investigated for the first time the effect of native and oxidized LDL with different subpatterns on endothelial cells of AA and CA, and those findings reveal the impact factors that may contribute to the different level of nitoxidative stress and endothelial dysfunction between endothelial cells of AA an CA.

4.3 Effects of LDL on ICAM-1 and VCAM-1 expression in HUVECs

4.3.1 Effects of n-LDL on ICAM-1 and VCAM-1 expression in HUVECs

In this study, we investigated the effect of different LDL patterns on upregulating ICAM-1/VCAM-1 expression in endothelial cells. ICAM-1 and VCAM-1 are cell adhesion molecules expressed in the membranes of endothelial cells. By using cell ELISA, we revealed that LDL stimulation significantly upregulated the expression of ICAM-1 and VCAM-1, leading to an increase of monocyte adhesion to endothelial cells. There is significant difference of ICAM-1/VCAM-1 expression between pattern A and B LDL stimulation. Pattern B LDL induced much higher levels of ICAM-1/VCAM-1 expression than pattern A, and pattern I stimulation is somewhere in between (Figure 3.27), suggesting that stimulation by pattern B LDL can enhance the highest adhesiveness of endothelial cells among those three subpatterns.

Our results are not consistent with a previous study taken by Takei and coworkers. They reported that native LDL at concentration of 100 µg/mL did not increase the expression of ICAM-1 and VCAM-1. It is possible that the concentration...
of LDL they used was too low and thus may not be sufficient to stimulate a significant change in ICAM-1/VCAM-1 expression compared to control. Activated endothelial cells may be involved in the development of atherosclerosis by interaction with LDL. Previous studies have shown that LDL can increase monocyte adhesion to endothelial cells by enhancing the expression level of ICAM-1 and/or VCAM-1.\textsuperscript{143, 144, 159-161} Our data are not only consistent with those reported results, but also further reveal that different subpatterns of LDL can upregulate distinctly the expression level of ICAM-1 and VCAM-1 to enhance adhesiveness of endothelial cells, which may attribute to the unique properties of LDL subpatterns, including but not limited to density, particle size, as well as components.

4.3.2 Effects of ox-LDL on ICAM-1 and VCAM-1 expression in HUVECs

We also investigated the effects of ox-LDL on upregulating ICAM-1 and VCAM-1 expression in HUVECs. Our data showed that the ox-LDL stimulated higher expression level of ICAM-1 and VCAM-1 than n-LDL (Figure 3.28 and 3.29), suggesting that ox-LDL is more likely to cause higher levels of monocyte adhesion to endothelial cells. However, our results are not consistent with the previously reported study. Wolfgang and colleagues found that incubation with ox-LDL at 100 µg/mL for 6 hours did not induce VCAM-1 expression, and it did not significantly upregulate ICAM-1 expression.\textsuperscript{144} It is possible that the concentration of ox-LDL they used for endothelial cell incubation may be too low to stimulate a significant increase for both ICAM-1 and VCAM-1 expression in endothelial cells. On the other hand, we used LDL subpatterns rather than general LDL during incubation, the potential interaction among different LDL subpatterns may impact
the expression of ICAM-1 and VCAM-1 distinctly from incubation with a single LDL subpattern. Among different ox-LDL subpatterns, pattern B still stimulated the highest level of ICAM-1/VCAM-1 expression, followed by pattern I; pattern A stimulated the lowest level of expression, which is still higher than control and n-LDL group.

Activated endothelial cells can produce ROS such as superoxide radical (·O$_2^-$), which can oxidize LDL and in turn ox-LDL causes enhancement of human blood monocytes adhesion to vascular endothelium.\textsuperscript{162} However, the mechanisms involved in this effect have not been completely elucidated yet. Our data from cell adhesion assay is consistent with ICAM-1 and VCAM-1 expression, suggesting that ox-LDL upregulated higher expression level of ICAM-1/VCAM-1, leading to more monocyte adhesion to endothelial cells, finally causing more severe damage to the cardiovascular system and triggered the adverse development of atherosclerosis.

4.3.3 Effects of n-LDL on ICAM-1 and VCAM-1 expression in HUVECs of African Americans and Caucasian Americans

In this study, we investigated the expression level of ICAM-1 and VCAM-1 by cell ELISA and compared the difference between endothelial cells of AA and CA. Cell ELISA result showed that the expression level of ICAM-1 and VCAM-1 was significantly increased after stimulation with n-LDL, suggesting that n-LDL upregulated the expression of ICAM-1 and VCAM-1 compared to control group. Meanwhile, the expression of ICAM-1 and VCAM-1 were higher in HUVECs of AA than in CA (for both control and LDL treatment group), suggesting that the higher expression level of ICAM-1 and VCAM-1 in AA than in CA not only resulted from the differences in race-
specific susceptibility to LDL stimulation, but also from the inherent high expression of ICAM-1 and VCAM-1 in HUVECs of AA. Data from ox-LDL incubation also confirmed this deduction. In summary, our study for the first time demonstrated the race-specific difference in the expression of ICAM-1 and VCAM-1 from HUVECs of AA and CA, and our data showed that HUVECs of AA were more susceptible to LDL stimulation than CA, thereby leading to the higher expression level of ICAM-1 and VCAM-1.

4.4 LDL induced cell adhesion in endothelial cells

4.4.1 n-LDL induced cell adhesion in endothelial cells

In this work, fluorescently pre-labeled THP-1 cells were used for monocyte adhesion assay. Our data showed that monocyte adhesion was time-dependent. As incubation time increased from 10 minutes to one hour, mean fluorescence intensity (MFI) increased as well, indicating that there were more monocytes adhering to endothelial cells as the incubation time increased. Our data also showed that monocyte adhesion was positive related to the concentration of LDL during incubation. Within the range of 50 to 400 µg/mL, MFI increased as concentration of LDL increased (Figure 3.35). At the same concentration, pattern B LDL stimulated the highest level of monocyte adhesion, while pattern A LDL stimulated the lowest level of monocyte adhesion. Control group showed a little increase in adhesion, indicating that iodixanol is not capable of stimulating monocyte adhesion as significant as LDL subpatterns.

LDL can stimulate the upregulation of ICAM-1 and VCAM-1 expression, which can further promote monocyte adhesion on endothelium surface, leading to the early lesion stage. Therefore, based on results from this study, pattern B LDL is the most
atherogenic among all of LDL subpatterns. The increase in monocyte adhesion resulted from interaction between LDL and endothelial cells. LDL heterogeneity leads to different subpatterns, the distinct compositional ratio of cholesterol, protein and triglyceride among LDL subpatterns may be associated with the different impacts on inducing monocyte adhesion to endothelial cells.

4.4.2 Ox-LDL induced cell adhesion in endothelial cells

As revealed in cell ELISA assay, ox-LDL upregulated higher expression levels of ICAM-1 and VCAM-1 than n-LDL, which can enhance monocyte adhesion to endothelial cells. In this study, ox-LDL stimulation induced more monocytes adhesion than n-LDL, which is consistent with cell ELISA results, indicating that ox-LDL is more atherogenic to the cardiovascular system than n-LDL by inducing more monocyte adhesion to endothelium. And this process may cause the development of pre-atherosclerosis.

However, the mechanism accounting for the different effect of ox-LDL and n-LDL on inducing monocyte adhesion is still unclear. Both chemical and physical changes in composition of LDL after oxidative modification may have a significant impact on increasing adhesiveness of endothelial cells and inducing monocyte adhesion. Ox-LDL can trigger the activation of NF-κB, which is a key factor contributing to the regulation of apoptosis, monocyte adhesion and inflammation. These results suggest that inhibiting both LDL oxidative modification and expression of adhesion molecules (ICAM-1 and VCAM-1) can reduce monocyte adhesion to endothelium, thereby it may provide a potential and effective way to develop a remedy for atherosclerosis.
4.4.3 n-LDL/ox-LDL induced cell adhesion in HUVECS of African Americans and Caucasian Americans

Our data showed that monocyte adhesion was drastically increased after incubation with n-LDL. Cell adhesion was higher in HUVECs of AA than in CA group (Figure 3.37), which is consistent with the results in cell ELISA assay. Blacks are considered as having higher risk of developing CVDs than Whites, but the mechanism that can explain this remains unclear. Our results provide direct evidence to reveal that the inherent high susceptibility to LDL stimulation in endothelial cells of AA leads to the enhancement of adhesiveness of endothelial cells, which may contribute to high levels of monocyte adhesion in arteries. Thus it can explain why Blacks have higher rates of cardiovascular morbidity and mortality than Whites. Furthermore, incubation with ox-LDL showed a similar result. The difference of monocyte adhesion between endothelial cells of AA and CA was more significant than control group (Figure 3.38), suggesting that the response of endothelial cells to n-LDL/ox-LDL stimulation is race-specific, resulting in higher monocyte adhesion on HUVECs of AA than CA, which may contribute to the development of atherosclerosis.
5 Conclusion

The concept of cholesterol has gone through several stages. Initially, high levels of cholesterol were related directly with high risk of CVDs. Later, it has been found that there are many subclasses of lipoproteins, and one of this subclass: LDL is bad, while another one: HDL is good. But it appears that this label is not accurate. There are several subpatterns of LDL, among which the small and dense LDL particles, pattern B, is the one that is in association with the development of CVDs. In this study, we demonstrate subpatterns of n-LDL/ox-LDL can alter NO and ONOO\(^{-}\) production differently, and this effect is dose-dependent. This is a main conclusion of our studies. The decrease in NO generation and increase in ONOO\(^{-}\) production suggest that pattern B may uncouple eNOS bioactivity more significantly than pattern I and A, and cause more severe dysfunction in endothelial cells. In addition, pattern B LDL not only stimulated higher expression of ICAM-1 and VCAM-1 than pattern I and A, but also induced the highest level of monocyte adhesion. We have also drawn a conclusion that pattern B LDL can impose more serious damage to endothelial cells than pattern A and I LDL, and the distribution of those three LDL patterns in human blood may play an important role in pathology of cardiovascular diseases. Studies on the effect and distribution of LDL patterns and their interaction with endothelium provide a fundamental molecular mechanism of LDL induced endothelial dysfunction and LDL triggered atherosclerosis.
Furthermore, we compared the effect of n-LDL/ox-LDL with different patterns on HUVECs of AA and CA, the effect of LDL subpatterns is more cytotoxic to endothelial cells of AA than CA. Apparently, a higher NO bioavailability and lower level of nitrooxidative stress in endothelial cells of CA compared to AA results in race-specific susceptibility of HUVECs to LDL stimulation. The \([\text{NO}]\) to \([\text{ONOO}^-]\) ratio was lower in endothelial cells of AA than in CA, in association with increased expression of ICAM-1 and VCAM-1, followed by higher monocyte adhesion in HUVECs of AA. This provides direct molecular evidences that endothelial dysfunction and the risk of cardiovascular complications are greater in AA than CA. The race differences in the distribution of LDL subpatterns and the metabolism of them in the human body remain unclear, and requires more study.
Figure 5.2 Race-specific difference in response to stimulation of different LDL subpatterns.
6 Future work

LDL with different patterns may play distinct roles in the cardiovascular system. In this study, we investigated the effect of LDL with each pattern on HUVECs as well as preliminary research based on LDL mixture with all patterns. However, more details about the distribution of LDL subpatterns in human plasma need to be studied, such as the factors which can impact this distribution including but not limited to age, gender, race, daily diet, smoking, obesity and so on. Future work may be focused on several directions. First, study could be carried out to investigate the effect of LDL mixtures with all patterns on stimulating the expression of ICAM-1 and VCAM-1 as well as monocyte adherence. In addition, it is necessary to measure NO and ONOO⁻ release from HUVECs stimulated by ox-LDL after incubation with multiple reagents such as L-Arg, PEG-SOD and L-NAME. Furthermore, future work could also be implemented to study the effect of LDL with multiple treatments such as L-Arg, PEG-SOD and L-NAME on stimulating the expression of ICAM-1 and VCAM-1 as well as monocyte adherence. Some drugs can decrease monocyte adhesion by inhibiting the expression of ICAM-1 and VCAM-1, thus it is worthy to investigate the expression of ICAM-1 and VCAM-1 as well as monocyte adhesion on endothelial cells after co-incubation of LDL with subpatterns and those drugs. All of the studies mentioned above are important and may provide potential benefits for establishing a standard protocol in clinical diagnosis and evaluating the risk of cardiovascular diseases.
References


Koppenol WH. The chemistry of peroxynitrite, a biological toxin. *Quim Nova.* 1998;21:326-331


Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: Implications for vascular endothelial function. *Biochemical and biophysical research communications.* 1999;263:681-684


Reynolds MR, Lukas TJ, Berry RW, Binder LI. Peroxynitrite-mediated tau modifications stabilize preformed filaments and destabilize microtubules through distinct mechanisms. *Biochemistry.* 2006;45:4314-4326


60. Salgo MG, Bermudez E, Squadrito GL, Pryor WA. Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes [corrected]. *Archives of biochemistry and biophysics*. 1995;322:500-505
63. Szabo C. Multiple pathways of peroxynitrite cytotoxicity. *Toxicology letters*. 2003;140-141:105-112

68. Apolipoproteins Mabtech.


73. Burke RMKaDJ. Indentification of multiple subclasses of plasma low density lipoproteins in normal humans. J. Lipid Res. 1982;23:97 - 104


82. Blake GJ, Otvos JD, Rifai N, Ridker PM. Low-density lipoprotein particle concentration and size as determined by nuclear magnetic resonance spectroscopy


93. Sparrow CP OJ. Cellular oxidation of low density lipoprotein is caused by thiol production in media containing transition metal ions. *Journal of lipid research*. 1993;34:1219-1228
97. Warnick GR, Knopp RH, Fitzpatrick V, Branson L. Estimating low-density lipoprotein cholesterol by the friedewald equation is adequate for classifying patients on the basis of nationally recommended cutpoints. *Clinical Chemistry.* 1990;36:15-19


128. Malinski T, Huk I. Measurement of nitric oxide in single cells and tissue using a porphyrinic microsensor. *Current protocols in neuroscience / editorial board, Jacqueline N. Crawley ... [et al.].* 2001;Chapter 7:Unit 7 14


133. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences of the United States of America.* 1990;87:1620-1624


