10840 J. Agric. Food Chem. 2010, 58, 10840–10846
DOI:10.1021/jf102120c

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Egg-Derived Peptide IRW Inhibits TNF-α-Induced Inflammatory Response and Oxidative Stress in Endothelial Cells

WUYANG HUANG,[†,§,||] SUBHADEEP CHAKRABARTI,[‡,||] KAUSTAV MAJUMDER,[†] YANYAN JIANG,[‡] SANDRA T. DAVIDGE,*[,‡] AND JIANPING WU*[,†]

[†]Department of Agricultural, Food and Nutritional Science, Faculty of Agricultural, Life and Environmental Sciences, University of Alberta, Edmonton, Alberta, Canada, [‡]Department of Obstetrics and Gynecology and Department of Physiology, Women's and Children's Health Research Institute, Cardiovascular Research Centre and the Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Alberta, Canada, and [§]Department of Functional Food and Bio-active Compounds, Institute of Agro-food Science and Technology, Jiangsu Academy of Agricultural Sciences, People's Republic of China. [||]Both authors contributed equally to this work.

Oxidative stress and vascular inflammatory response are key mediators of endothelial dysfunction that leads to cardiovascular diseases. A novel peptide, IRW, was previously characterized from egg protein with angiotensin converting enzyme inhibitory activity. The purpose of the study was to investigate the effects and molecular mechanisms of IRW on regulating inflammatory response in endothelial cells. The results showed that tumor necrosis factor-α (TNF-α) significantly increased the protein levels of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and monocyte chemotactic protein-1 (MCP-1), whereas pretreatment with IRW inhibited TNF-α-induced increases of ICAM-1, VCAM-1, and MCP-1 production in a concentration-dependent manner. IRW also reduced the levels of superoxide ions in the presence and absence of TNF-α. These results indicate the potential role of IRW in preventing cardiovascular disease as a functional food ingredient or nutraceutical.

KEYWORDS: Egg peptide IRW; anti-inflammatory; antioxidant; endothelial cells; tumor necrosis factor-α (TNF-α)

INTRODUCTION

Cardiovascular diseases are the leading cause of death and disability worldwide (*1*). Oxidative stress is a major cause of reduced endothelial nitric oxide availability in hypertension, one of the well-defined risk factors for cardiovascular diseases. The vascular inflammatory response plays an important role in the formation of an atherosclerotic plaque (*2*). Tumor necrosis factor-α (TNF-α), a prototypic pro-inflammatory cytokine found in atherosclerotic lesions, can have direct effects on vascular endothelial cells to induce the expression of cell adhesion molecules and to facilitate the infiltration of leukocytes (*3*). Inflamed endothelial cells up-regulate monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), etc., which are important in the onset and progress of inflammation (*3*−*5*).

MCP-1 is a potent chemoattractant for monocytes, basophils, and memory T cells. MCP-1 is one of the key factors involved in the initiation of inflammation and is involved in the pathogenesis of cardiovascular diseases (*6*). Previous studies on increased MCP-1 mRNA expression in macrophage-rich atherosclerotic plaques suggested MCP-1 could mediate monocytic infiltration of the artery wall (*7*). Because MCP-1 is implicated in multiple inflammatory diseases, developing a therapy that targets MCP-1 up-regulation may be an important strategy against inflammatory conditions.

ICAM-1 and VCAM-1 are two adhesion molecules, belonging to members of the immunoglobulin (Ig) superfamily, up-regulated during inflammatory responses (*8*). Adhesion molecules are essential for transmigration of monocytes through the vascular wall into the intima, where they take up oxidized cholesterol and accumulate as foam cells (*8*). ICAM-1 is important in stabilizing cell−cell interactions and facilitating leukocyte endothelial transmigration. ICAM-1 is present in low levels on the membranes of resting endothelial cells. Upon cytokine stimulation, such as TNF-α, these concentrations greatly increase and lead to leukocyte recruitment (*5*). VCAM-1 is up-regulated in cytokine-activated endothelium. VCAM-1 mediates leukocyte-endothelial cell adhesion and signal transduction and may play a role in the development of atherosclerosis and rheumatoid arthritis (*3*). VCAM-1 promotes the adhesion of lymphocytes, monocytes, eosinophils, and basophils to the activated endothelial cells (*9*). The regulation of interaction mediated by these adhesion molecules may provide a new target for controlling inflammatory and immune responses.

*Address correspondence to either author [(S.T.D.) 232 HMRC, University of Alberta, Edmonton, AB, Canada T6G 2S2 (phone (780) 492-1864, fax (780) 492-1308, e-mail Sandra.davidge@ualberta.ca); (J.W.) 4-10 Ag/For Centre, Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada T6G 2P5 (phone (780) 492-6885, fax (780) 492-4265, e-mail jianping.wu@ualberta.ca)].

 Published on Web 10/01/2010 © 2010 American Chemical Society

The pro-inflammatory nuclear factor κB (NF-κB) is recognized as one major transcription factor that mediates key steps in the development of atherosclerotic lesions (*3*). In fact, NF-κB can be rapidly activated by many pathogenic stimuli, such as TNF-α and reactive oxygen species (*6*). IκBα and IκBβ are cytoplasmic NF-κB regulatory proteins. Prior to cytokine stimulation, NF-κB is restricted to the cytosol as an inactive complex with its inhibitors such as IκBα. Upon activation by pro-inflammatory cytokines, IκBα is phosphorylated by IκB protein kinase, rapidly degraded, and dissociated from the NF-κB. Activated NF-κB enters the nucleus and binds κB consensus regulatory elements in the promoters of many responsive genes (*3*, *10*). The regulation of NF-κB also may provide a new strategy against inflammatory and immune responses.

Due to the inevitable side effects of synthetic drugs, there is an interest in seeking novel bioactive food components, such as angiotensin converting enzyme (ACE) inhibitory peptides, for the prevention and treatment of cardiovascular diseases (*11*). Food-derived bioactive peptides are reported to have a wide range of bioactivities including antimicrobial, anticarcinogenic, anti-inflammatory, antioxidant, and antihypertensive effects (*12*). Using an integrated quantitative structure and activity relationship (QSAR) and bioinformatics approach, a novel ACE inhibitory peptide, Ile-Arg-Trp (IRW), was characterized from ovotransferrin (*13*). Because angiotensin II is a pro-inflammatory compound that can induce the production of reactive oxygen species, an ACE inhibitor might contribute to anti-inflammatory activity (*14*). The objective of the study was to study the antioxidant or anti-inflammatory properties of this peptide in human vascular umbilical endothelial cells.

MATERIALS AND METHODS

Chemicals and Reagents. Dulbecco's phosphate-buffered saline (PBS), M199 medium with phenol red, TNF-α, glutamine, porcine gelatin, and heparin were bought from Sigma Chemical Co. (St. Louis, MO). M199 medium without phenol red, fetal bovine serum (FBS), and L-glutamine were obtained from Gibco/Invitrogen (Carlsbad, CA). The antibiotics penicillin and streptomycin were purchased from Life Technologies (Carlsbad, CA). Type 1 collagenase was obtained from Worthington Biochemical Corp. (Lakewood, NJ). Triton X-100 and endothelial cell growth supplement (ECGS) were obtained from VWR International (West Chester, PA). Dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR). IRW was synthesized by GenScript Corp. (Piscataway, NJ), and its purity (>95%) was verified by HPLC-MS/MS. All of the chemicals and reagents were of analytical grade.

Antibodies. Mouse monoclonal primary antibody against ICAM-1, rabbit polyclonal primary antibodies against VCAM-1, the p65 component of NF-κB, and TNF receptor 1 (TNFR1) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and used at a concentration of 1 μg/mL for Western blots. Rabbit polyclonal primary antibody to TNF receptor 2 (TNFR2) and the α-tubulin antibody were bought from Abcam (Cambridge, MA), which were used at 1 and 0.4 μg/mL, respectively. Rabbit monoclonal primary antibody to IκBα and rabbit polyclonal primary antibody to IκBβ were from Cell Signaling Technology Inc. (Danvers, MA) and both used at 1:1000 dilutions. Donkey anti-mouse and goat anti-rabbit HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA) and used at 1:5000 dilutions.

Endothelial Cell Culture and Treatment. Human umbilical vein endothelial cells (HUVECs) have been well characterized as a model system for studying inflammation and oxidative stress in the vasculature (*15*). The protocols were approved by the University of Alberta Ethics Committee, and the studies were conducted according to the principles of the Declarations of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, revised November 13, 2001, effective December 13, 2001. All subjects provided informed consent prior to inclusion in this study. HUVECs were isolated from umbilical cords kindly provided by the Royal Alexandra Hospital in Edmonton, AB, Canada. Second passage cells were used for all experiments.

On the day of the experiment, confluent monolayers (at 80−90% confluence) of second-passage HUVECs were quiesced in a reduced serum medium (phenol-free M199 media with 1% FBS, 2 mmol/L L-glutamine, and 1% penicillin−streptomycin) for 4 h prior to the start of the actual experiment. In a separate set of experiments, the cells were treated with egg-derived peptide IRW for 24 h with or without TNF-α stimulation (5 ng/mL for 6 h). At the end of the specified incubation period, the supernatants were collected, centrifuged, and stored at −80 °C until further MCP-1 analysis. The cells were lysed in boiling hot Laemmli's buffer containing 0.2% Triton-X-100 to prepare samples for Western blotting.

Human Monocyte Chemoattractant Protein-1 Analysis. The levels of immunoreactive human MCP-1 were quantified from the cell-free endothelial supernatants using a solid phase sandwich ELISA kit (Biosource International Inc., Camarillo, CA). Briefly, samples and standards were piptetted into the wells of the microtiter strips, which had been coated with a monoclonal antibody specific for human MCP-1, followed by the addition of a biotinylated polyclonal second antibody. The assay procedure was employed according to the kit protocol booklet instructions. The samples were conducted with protein assay and suitably diluted in the standard diluent buffer. The absorbance of the resulting yellow color was measured at 450 nm on an EL 808 (Bio-Tek Instruments Inc., Winooski, VT) microplate reader. The reader was controlled via KC junior (version 1.6, Bio-Tek) software.

Western Blotting. Western blotting was performed on the HUVEC lysates as described before (*16*). The protein bands were detected by a Fluor-S-Max multi-imager and quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA). Data were normalized by reprobing the membrane with an antibody against α-tubulin, which was used as a loading control. Samples generated from a particular umbilical cord were run on the same gel. Cell lysates from untreated cells were loaded on every gel, and all data were expressed as fold increase over the corresponding untreated control (no IRW or TNF-α).

Immunofluorescence. HUVECs were fixed in 3.75% paraformaldehyde, permeabilized with 0.1% Triton X-100 incubated overnight with primary antibody against p65. On the following day, the cells were treated with Alexa Fluor 546 conjugated goat anti-rabbit secondary antibody (Molecular Probes) for 30 min in the dark. The cell nuclei were stained with Hoechst 33342 dye from Molecular Probes. Cells were visualized under an Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group, Ontario, Canada) using Slidebook 2D, 3D Timelapse Imaging software (Intelligent Imaging Innovations Inc., Denver, CO). All images presented are in ×100 magnification.

Superoxide Detection Assay. Endothelial superoxide generation was measured by staining with DHE. DHE is cell permeable and reacts with superoxide to yield ethidium, which binds to nuclear DNA and generates nuclear fluorescence (*17*). Briefly, HUVEC monolayers were washed once and incubated for 30 min at room temperature with 10 μM DHE in the reduced serum medium. At the end of this incubation period, cells were washed once and fluorescence was visualized in a fluorescence microscope (Olympus IX81, Olympus Canada Inc., Ontario, CA) under Cy3 (red) channel. For each data point, images from three randomly chosen fields were taken. The total fluorescence intensity and the number of cells in each field were noted, and the mean fluorescence intensity per cell (MFI/cell) was determined similarly to a previously reported method (*18*). Superoxide generation was measured as fold increase in MFI/cell over the untreated control.

Statistical Analysis. All data presented are mean value ± SEM of between four and five independent experiments using HUVECs isolated from different umbilical cords. Data are expressed as fold change over the untreated control. One-way analysis of variance with an appropriate post test was used for the determination of statistical significance. Dunnett's post test for comparison with control and Bonferroni's post test for multiple comparisons were used. A repeated measures test was used whenever applicable. Differences were considered to be significant with a P value of < 0.05.

RESULTS

Effects of IRW on TNF-α-Induced MCP-1 Production. To determine whether TNF-α-stimulated MCP-1 production is affected by IRW, endothelial cells were pretreated for 18 h with

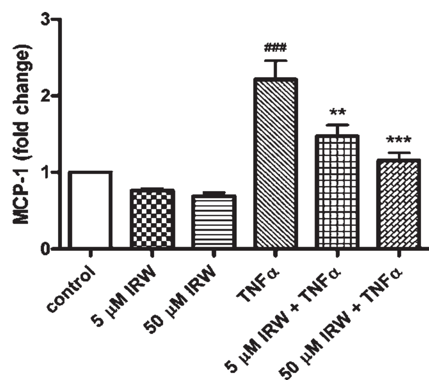

Figure 1. Effects of IRW concentrations on TNF-α-mediated changes in MCP-1 production released into the medium. Confluent HUVEC monolayers were pretreated for 18 h with different concentrations of IRW prior to 6 h of incubation with 5 ng/mL TNF-α. MCP-1 levels are expressed as fold increase over the untreated control. Bars represent mean values ± SEM, $n = 5$ separate experiments. ### indicates $P < 0.001$ as compared to control; ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively, compared to TNF-α alone.

0−50 μM IRW prior to TNF-α (5 ng/mL) stimulation for 6 h. IRW alone did not alter basal MCP-1 level; however, IRW significantly inhibited TNF-α-induced MCP-1 production in a concentration-dependent manner (**Figure 1**). IRW at 5 and 50 μM could inhibit 62.6 and 87.3% of TNF-α-increased protein levels of MCP-1, respectively. These data suggested an interaction between these two different factors on the regulation of endothelial MCP-1 expression.

Effects of IRW on TNF-α-Induced ICAM-1 and VCAM-1 Protein Expression. Similar to MCP-1, 5 ng/mL TNF-α significantly increased the expression of ICAM-1 and VCAM-1 in endothelial cells (**Figure 2**). The protein level of ICAM-1 showed about a 4-fold increase over the untreated control, whereas that of VCAM-1 showed a >6-fold increase over the untreated control. Various levels of IRW alone had little effect on the protein levels of endothelial ICAM-1 and VCAM-1. IRW at 5 μM could just inhibit 16.3 and 35.7% of TNF-α-induced ICAM-1 and VCAM-1 levels, whereas 50 μM IRW could significantly alter TNF-α-induced ICAM-1 and VCAM-1 levels ($P < 0.05$), with inhibitions of 72.1 and 80.0%, respectively. These data indicate that IRW actually inhibited expression of adhesion molecules ICAM-1 and VCAM-1 induced by TNF-α in endothelium.

Effects of IRW on TNF-α-Induced TNFR1 and TNFR2 Protein Expression. TNF-α exerts its effects through two distinct receptors, TNFR1 and TNFR2 (*19*). To understand the potential mechanisms for IRW inhibiting endothelial inflammatory responses induced by TNF-α, we further investigated whether IRW could alter the expression of TNFR1 and TNFR2 in endothelial cells. A concentration of 50 μM IRW alone did not affect TNFR1 and TNFR2 expression. After 6 h of TNF-α stimulation treatment, TNFR1 was significantly decreased to 56.3% compared to control ($P < 0.05$, **Figure 3A**). In contrast, on pretreatment with 50 μM IRW before the addition of TNF-α, TNFR1 was decreased to 71.6% of control (inhibition = 35%). It seemed that IRW could partly prevent TNF-α-induced TNFR1 down-regulation; however, the change of TNFR1 level with and without IRW is not significant. In addition, TNF-α or IRW could not alter TNFR2 levels (**Figure 3B**).

Effects of IRW on TNF-α-Induced IκB Degradation. Our preliminary experiment (to treat cells with TNF-α for 15 min, 30 min, 1 h, or 2 h) showed that treatment with TNF-α for 30 min showed the greatest change of IκBα levels, whereas the levels of IκBβ were not consistently altered (data not shown). To determine whether the inflammation inhibitory effects of IRW correlated with effects on the cytoplasmic NF-κB regulatory proteins, IκBα and IκBβ, cells were preincubated with IRW for 18 h, and with or without TNF-α for 30 min thereafter. The amount of IκBα was greatly reduced after exposure to the cytokine TNF-α. However, the degradation of IκBα was not significantly affected by IRW. Neither TNF-α nor IRW altered IκBβ levels (**Figure 4**). Therefore, IRW appears to possess IκB-independent anti-inflammatory mechanism of action.

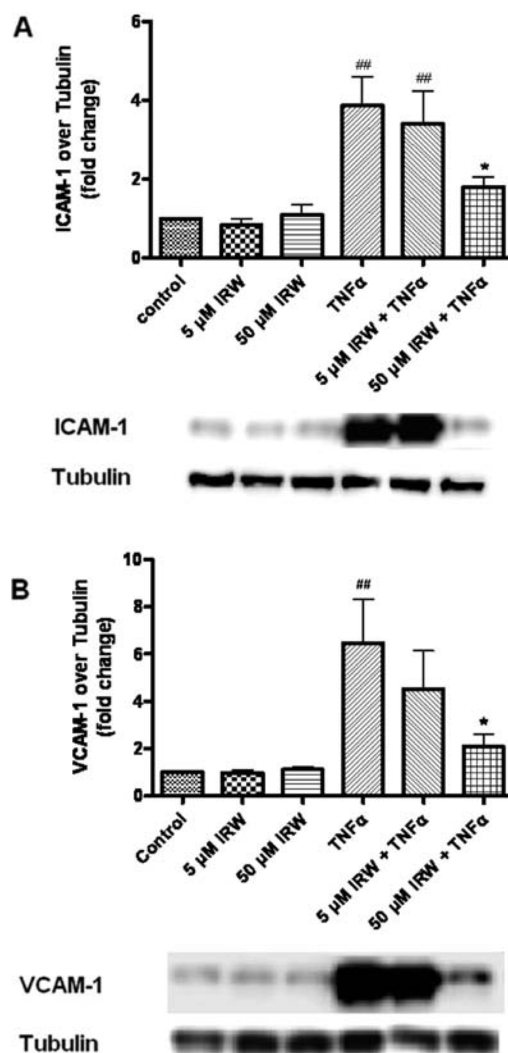

Figure 2. Effects of IRW inhibition on TNF-α-induced ICAM-1 (**A**) and VCAM-1 (**B**) protein expression. Confluent HUVEC monolayers were pretreated for 18 h with IRW prior to 6 h of incubation with 5 ng/mL TNF-α. ICAM-1 and VCAM-1 protein levels are expressed as fold increase over the untreated control. Bars represent mean values ± SEM, $n = 4-5$ separate experiments. Representative Western blots are shown. ## indicates $P < 0.01$ as compared to control; * indicates $P < 0.05$ compared to TNF-α alone.

Effects of IRW on TNF-α-Induced NF-κB Translocation. On activation of the NF-κB pathway, the p65 and p50 proteins are released from the cytosol and migrate into the cell nucleus, where they interact with the promoter regions of various proteins that are up-regulated in inflammation. Thus, nuclear translocation of p65 is widely used as a measure for NF-κB activation. HUVECs were treated with IRW for 18 h and with or without TNF-α for 30 min thereafter. TNF-α stimulation caused nuclear translocation of p65. Interestingly, pretreatment with IRW abolished the

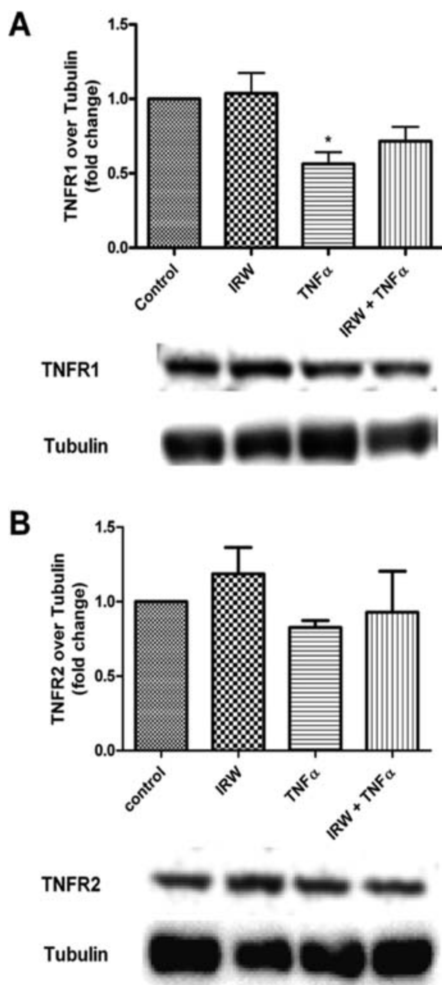

Figure 3. Effects of IRW on TNF-α receptors (TNFR1, **A**; TNFRII, **B**) protein expression. Confluent HUVEC monolayers were pretreated for 18 h with 50 μM of IRW prior to 6 h of incubation with 5 ng/mL TNF-α. TNFR1 and TNFRII protein levels are expressed as fold of the untreated control. Bars represent mean values ± SEM, n = 4 separate experiments. $*$ indicates $P < 0.05$ as compared to control.

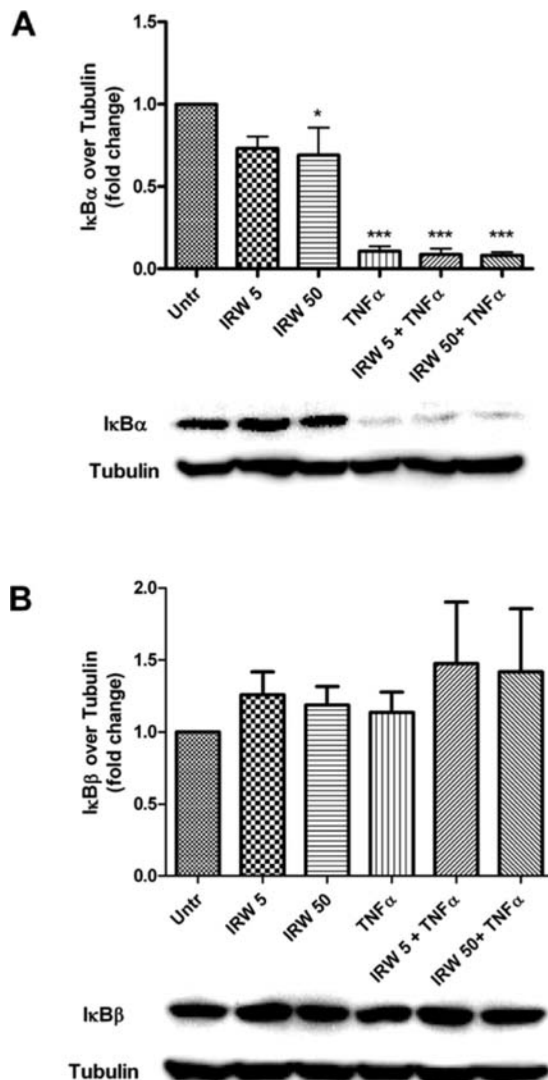

Figure 4. Effects of IRW on IκBα (**A**) and IκBβ (**B**) protein levels in endothelial cells. Confluent HUVEC monolayers were pretreated for 18 h with different concentrations of egg-derived peptide IRW (0, 5, 50 μM) prior to 30 min of incubation with 5 ng/mL of TNF-α. IκBα and IκBβ protein levels are expressed as fold of the untreated control. Bars represent mean values ± SEM, n = 4−5 separate experiments. $*$ and $***$ indicate $P < 0.05$ and $P < 0.001$, respectively, compared to control.

TNF-α induced nuclear localization of p65, suggesting a key role for IRW at this critical step in the NF-κB pathway (**Figure 5**).

Effects of IRW on TNF-α-Mediated Superoxide Generation. Inflammatory responses are often associated with increased oxidative stress. Therefore, the effect of IRW on the superoxide generation in endothelial cells was studied. Our results showed that 50 μM IRW alone could slightly decrease the levels of superoxide ions in cells, which was down to 80.9% of the untreated control. Superoxide generation in HUVEC increased with exposure to 5 ng/mL TNF-α for 6 h, but was significantly abrogated by prior treatment with IRW for 18 h. The levels of superoxide ions from TNF-α-mediated cells with and without IRW treatment were 96.2 and 130.9%, respectively (**Figure 6**). There was a significant difference between them ($P < 0.001$). These data suggest that IRW possessed antioxidant capacity by abrogation of the superoxide-induced oxidative stress.

DISCUSSION

Many food sources contribute to cardiovascular health, such as fruits, vegetables, cereals, legumes, fish, milk, and tea, as well as eggs (*11*). Eggs are economically and nutritionally important because they can form a significant component of the diet and they are also an excellent source of bioactive substances (*12*). Egg protein derived bioactive peptides were reported to have antioxidant, anti-inflammatory, and angiotensin converting enzyme (ACE) inhibitory properties (*12*). These indicate that egg-derived peptides could be the potential alternatives used to prevent and treat cardiovascular diseases. The bioactive peptide IRW used in this study was first characterized as a potent ACE inhibitory peptide from ovotransferrin, an egg white protein with a potent superoxide anion scavenging activity as a superoxide dismutase mimic protein (*14*, *20*). Our previous studies found IRW possessed high oxygen radical absorbance capacity in vitro (*21*). In the present study, IRW also showed antioxidant and anti-inflammatory properties in isolated endothelial cells.

Vascular inflammation, mediated through pro-inflammatory cytokines such as TNF-α, plays key roles in the pathogenesis of atherosclerosis and its complications. MCP-1 is a chemokine upregulated in response to TNF-α stimulation involved in vascular pathology. MCP-1 expression has been observed in many tissues during inflammation-dependent disease progression, including atherosclerosis (*22*). Therefore, MCP-1 is important in vascular

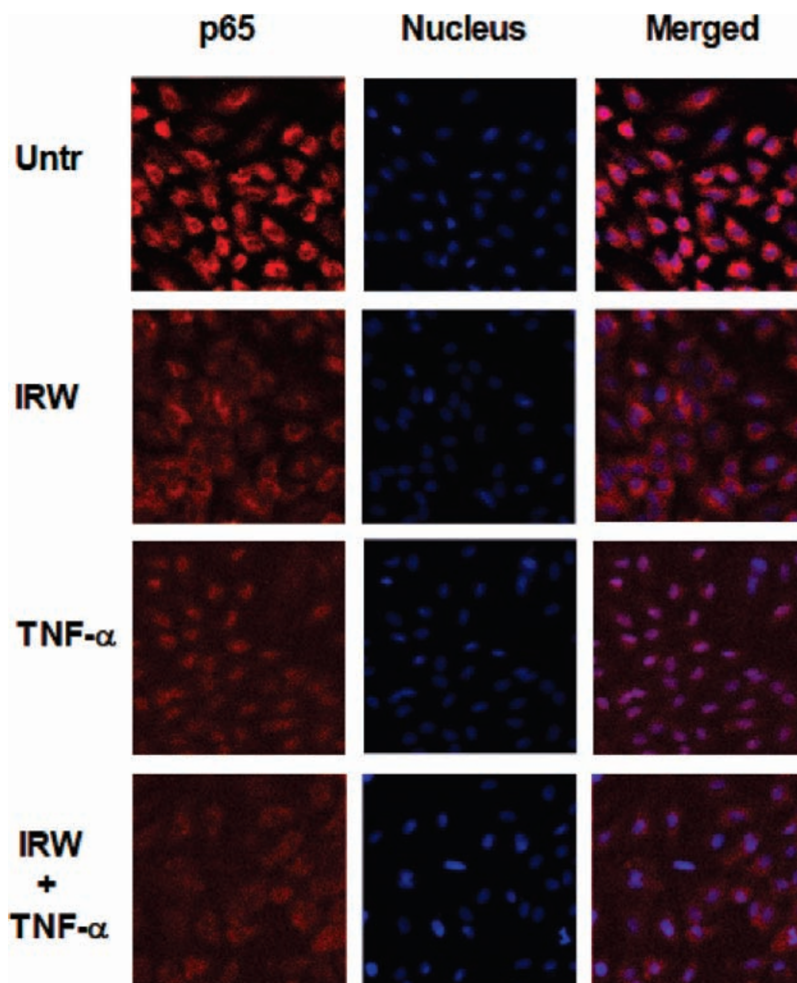

Figure 5. Effects of IRW and TNF-α on endothelial p65 translocation. Confluent HUVEC monolayers were pretreated for 18 h with 50 μM IRW prior to 30 min of incubation with 5 ng/mL TNF-α. Cells were fixed, permeabilized, and immunostained for p65. A representative set of images from three independent experiments is shown.

complications, and the development of specific inhibitors of this chemokine represents a novel strategy for the treatment of inflammatory diseases. In the present study, we found that IRW prevented TNF-α-induced MCP-1 overexpression.

ICAM-1 and VCAM-1 are important in inflammatory responses and play crucial roles in cell adhesion and cell signal transduction (*3*). Cell−cell interactions mediated by intercellular adhesion molecules have fundamental function during tissue morphogenesis in embryonic development, as well as metastatic invasion of tumor cells. Both ICAM-1 and VCAM-1 play roles in the progression of various diseases such as atherosclerosis (*3*). ICAM-1 and VCAM-1 were overexpressed in vascular endothelial cells when exposed to TNF-α (*5*). In this study, we confirmed that TNF-α significantly increased ICAM-1 and VCAM-1 levels in HUVECs. However, pretreatment with a high level of IRW significantly blocked TNF-α-induced overexpression of both ICAM-1 and VCAM-1. These two adhesion molecules in endothelial cells have been demonstrated to actively participate in leukocyte migration from the blood into tissues, which is vital for immune surveillance and inflammation (*23*). ICAM-1 ligation produces pro-inflammatory effects such as inflammatory leukocyte recruitment by signaling though cascades involving many kinases, such as the src-kinase p53/p56lyn (*24*). The inhibition of TNF-α stimulated ICAM-1 and VCAM-1 expression indicated that IRW possessed potential pharmaceutical use in inflammatory-related diseases. To the best of our knowledge, this is the first time an egg-derived peptide has been shown to modulate inflammatory responses in the endothelium.

TNF-α exerts multiple biological effects, including induction of leukocyte adhesion molecules and pro-inflammatory cytokines, fibrin deposition, alterations of endothelial cytoskeletal actin, formation of intercellular gaps, regulation of vascular permeability, and modulation of nitric oxide production (*25*). These processes are critical in the physiological response to several inflammatory conditions and in a variety of pathological conditions. TNF-α has two receptors in the cell membrane. TNFR1 signals both inflammation and apoptosis, whereas TNFR2 primarily mediates cell survival (*19*). TNF-α induces a redistribution and down-regulation of TNFR1 in endothelial cells (*26*). IRW only weakly changed the level of TNF-α-induced TNFR1 reduction (inhibition = 35%, but no significant difference) in the present study. Similarly to a previous study (*4*), TNFR2 was not altered either. These data suggest that egg peptide IRW attenuated TNF-α-induced inflammatory responses and oxidative stress without interfering with the TNF receptors. This indicates a potential role for alterations involving the downstream signaling cascades in mediating the anti-inflammatory and antioxidant effects of IRW.

The increase in NF-κB activity is well correlated with mononuclear cell infiltration and expression of MCP-1 and adhesion molecules (e.g., ICAM-1 and VCAM-1) (*10*). NF-κB activation could be regulated by cytoplasmic proteins IκBα and IκBβ. IκBα

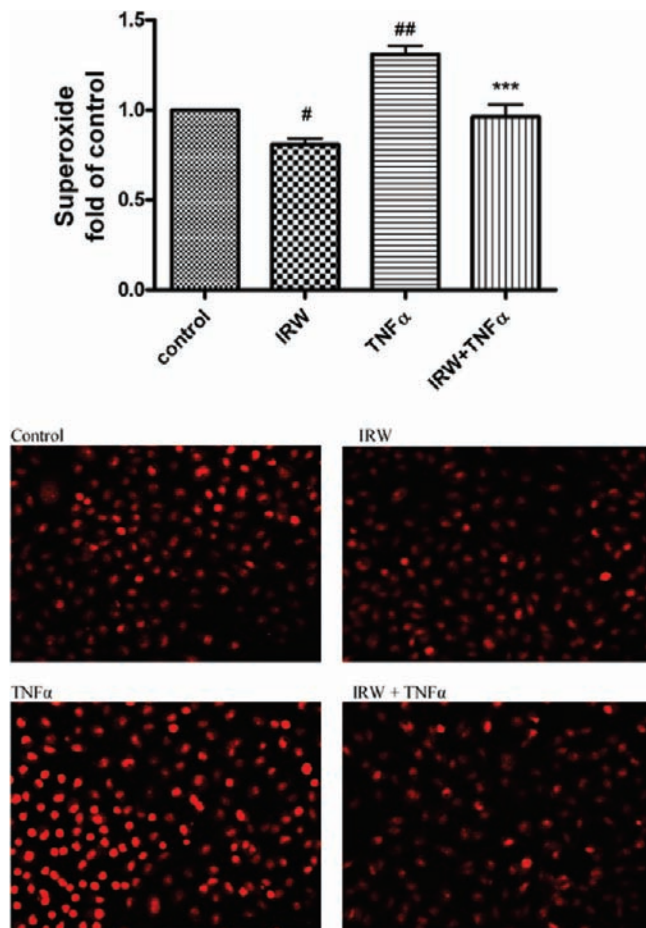

Figure 6. Effects of IRW and TNF-α on endothelial superoxide generation. Confluent HUVEC monolayers were pretreated for 18 h with 50 μM IRW prior to 6 h of incubation with 5 ng/mL TNF-α. A representative set of images is shown. Data were calculated as MFI/Cell and expressed as fold increase over the untreated control. Bars represent mean values ± SEM, $n = 4$ separate experiments. # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, compared to control; *** indicates $P < 0.001$ compared to TNF-α alone.

inhibits NF-κB activity by forming an inactive complex (*3*). Secretory leukocyte protease inhibitor blocked NF-κB activation by elevating the IκBβ level during lung inflammation (*27*). We found TNF-α caused rapid degradation of IκBα, whereas neither TNF-α nor IRW affected the levels of IκBβ. The fact that IRW did not alter the TNF-α-induced degradation of IκBα suggests the existence of an IRW-modulated, IκB-independent mechanism. Recent data have shown that the mitogen-activated protein kinase (MAPK) family is indeed involved in IκB independent NF-κB activation (*28*). Interestingly, IRW was able to inhibit the nuclear translocation of p65, suggesting a novel mechanism by which this egg peptide could block pro-inflammatory signaling downstream of IκB in this study.

Because ACE inhibitory IRW had broad effects in inflammatory conditions by preventing TNF-α mediated events, we speculated that IRW could ameliorate cellular oxidative stress. The results showed that IRW decreased superoxide generation in the presence or absence of TNF-α. Previous studies also found that the ACE inhibitory dipeptide Met-Tyr could diminish free radical formation in human endothelial cells (*29*). As noted before, increased oxidative stress plays a key role in the pathogenesis of cardiovascular diseases (*2*). Therefore, IRW has the potential benefit of preventing cardiovascular diseases by ameliorating oxidative stress. Moreover, reactive oxygen species may act as signal transduction messengers for several important transcription factors and play a major role in the inflammatory activation of NF-κB (*6*). It was conceivable that antioxidant actions might have accounted for the anti-inflammatory effects of IRW.

This study demonstrated that IRW attenuated TNF-α-induced inflammatory responses and oxidative stress in endothelial cells. This indicated the potential role of IRW as a functional food ingredient or nutraceutical in preventing cardiovascular disease. Further investigations in progress are to demonstrate in vivo properties in animals and the regulation of the pathways, which will lead to a better understanding of the protective mechanisms of egg-derived peptide IRW on the vascular system given the importance of both oxidative stress and inflammation on vascular pathologies.

ABBREVIATIONS USED

ACE, angiotensin converting enzyme; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular cell adhesion molecule-1; IκB, inhibitor κB; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

ACKNOWLEDGMENT

We thank Donna Dawson, Nurse Research Coordinator in the Royal Alexandra Hospital, Edmonton, AB, for providing human umbilical cords.

LITERATURE CITED

(1) Bonow, R. O.; Smaha, L. A.; Smith, S. C., Jr.; Mensah, G. A.; Lenfant, C. World Heart Day 2002 − the international burden of cardiovascular disease: responding to the emerging global epidemic. *Circulation* **2002**, *106*, 1602−1605.
(2) Landmesser, U.; Harrison, D. G. Oxidant stress as a marker for cardiovascular events: Ox marks the spot. *Circulation* **2001**, *104*, 2638−2640.
(3) Chai, H.; Wang, Q.; Huang, L.; Xie, T.; Fu, Y. Ginsenoside Rb1 inhibits tumor necrosis factor-α-induced vascular cell adhesion molecule-1 expression in human endothelial cells. *Biol. Pharm. Bull.* **2008**, *31*, 2050−2056.
(4) Ahn, H. Y.; Xu, Y.; Davidge, S. T. Epigallocatechin-3-*O*-gallate inhibits TNF-α-induced monocyte chemotactic protein-1 production from vascular endothelial cells. *Life Sci.* **2008**, *82*, 964−968.
(5) Yang, L.; Froio, R. M.; Sciuto, T. E.; Dvorak, A. M.; Alon, R.; Luscinskas, F. W. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-α-activated vascular endothelium under flow. *Blood* **2005**, *106*, 584−592.
(6) Melgarejo, E.; Medina, M. A.; Sanchez-Jimenez, F.; Urdiales, J. L. Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 998−1001.
(7) Yu, X.; Dluz, S.; Graves, D. T.; Zhang, L.; Antoniades, H. N.; Hollander, W.; Prusty, S.; Valente, A. J.; Schwartz, C. J.; Sonenshein, G. E. Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6953−6957.
(8) Sudano, I.; Spieker, L. E.; Hermann, F.; Flammer, A.; Corti, R.; Noll, G.; Luscher, T. F. Protection of endothelial function: targets for nutritional and pharmacological interventions. *J. Cardiovasc. Pharmacol.* **2006**, *47*, S136−150.
(9) Verdegaal, E. M.; Beekhuizen, H.; Blokland, I.; van Furth, R. Increased adhesion of human monocytes to IL-4-stimulated human venous endothelial cells via CD11/CD18, and very late antigen-4 (VLA-4)/vascular cell adhesion molecule-1 (VCAM-1)-dependent mechanisms. *Clin. Exp. Immunol.* **1993**, *93*, 292−298.
(10) Lentsch, A. B.; Jordan, J. A.; Czermak, B. J.; Diehl, K. M.; Younkin, E. M.; Sarma, V.; Ward, P. A. Inhibition of NF-κB activation and augmentation of IκBβ by secretory leukocyte protease inhibitor during lung inflammation. *Am. J. Pathol.* **1999**, *154*, 239−247.

(11) Chen, Z. Y.; Peng, C.; Jiao, R.; Wong, Y. M.; Yang, N.; Huang, Y. Anti-hypertensive nutraceuticals and functional foods. *J. Agric. Food Chem.* **2009**, *57*, 4485−4499.

(12) Mine, Y. Egg proteins and peptides in human health − chemistry, bioactivity and production. *Curr. Pharm. Des.* **2007**, *13*, 875−884.

(13) Majumder, K.; Wu, J. A new approach for identification of novel antihypertensive peptides from egg proteins by QSAR and bioinformatics. *Food Res. Int.* **2010**, *43*, 1371−1378.

(14) Mertens, B.; Vanderheyden, P.; Michotte, Y.; Sarre, S. The role of the central renin-angiotensin system in Parkinson's disease. *J. Renin Angiotensin Aldosterone Syst.* **2010**, *11*, 49−56.

(15) Finkenzeller, G.; Graner, S.; Kirkpatrick, C. J.; Fuchs, S.; Stark, G. B. Impaired in vivo vasculogenic potential of endothelial progenitor cells in comparison to human umbilical vein endothelial cells in a spheroid-based implantation model. *Cell Prolif.* **2009**, *42*, 498−505.

(16) Stewart, K. G.; Zhang, Y.; Davidge, S. T. Estrogen decreases prostaglandin H synthase products from endothelial cells. *J. Soc. Gynecol. Investig.* **1999**, *6*, 322−327.

(17) Peshavariya, H. M.; Dusting, G. J.; Selemidis, S. Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase. *Free Radical Res.* **2007**, *41*, 699−712.

(18) Dyugovskaya, L.; Lavie, P.; Lavie, L. Increased adhesion molecules expression and production of reactive oxygen species in leukocytes of sleep apnea patients. *Am. J. Respir. Crit. Care Med.* **2002**, *165*, 934−939.

(19) D'Alessio, A.; Al-Lamki, R. S.; Bradley, J. R.; Pober, J. S. Caveolae participate in tumor nercrosis factor receptor 1 signaling and internalization in human endothelial cell line. *Am. J. Pathol.* **2005**, *166*, 1273−1282.

(20) Ibrahim, H. R.; Hoq, M. I.; Aoki, T. Ovotransferrin possesses SOD-like superoxide anion scavenging activity that is promoted by copper and manganese binding. *Int. J. Biol. Macromol.* **2007**, *41*, 631−640.

(21) Huang, W. Y.; Majumder, K.; Wu, J. Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interactions with phytochemicals. *Food Chem.* **2010**, *123*, 635−641.

(22) O'Hayre, M.; Salanga, C. L.; Handel, T. M.; Allen, S. J. Chemokines and cancer: migration, intracellular signaling and intercellular communication in the microenvironment. *Biochem. J.* **2008**, *409*, 635−649.

(23) Cook-Mills, J. M.; Deem, T. L. Active participation of endothelial cells in inflammation. *J. Leukocyte Biol.* **2005**, *77*, 487−495.

(24) Lawson, C.; Wolf, S. ICAM-1 signaling in endothelial cells. *Pharmacol. Rep.* **2009**, *61*, 22−32.

(25) Ferrero, E.; Zocchi, M. R.; Magni, E.; Panzeri, M. C.; Curnis, F.; Rugarli, C.; Ferrero, M. E.; Corti, A. Roles of tumor necrosis factor p55 and p75 receptors in TNF-α-induced vascular permeability. *Am. J. Physiol. Cell Physiol.* **2001**, *281*, C1173−1179.

(26) Gupta, S. A decision between life and death during TNF-α-induced signaling. *J. Clin. Immunol.* **2002**, *22*, 185−194.

(27) Ruiz-Ortega, M.; Lorenzo, O.; Egido, J. Angiotensin III increases MCP-1 and activates NF-κB and AP-1 in cultured mesangial and mononuclear cells. *Kidney Int.* **2000**, *57*, 2285−2298.

(28) Huh, J.-E.; Yim, J.-H.; Lee, H.-K.; Moon, E.-Y.; Rhee, D.-K.; Pyo, S. Prodigiosin isolated from *Hahella chejuensis* suppresses lipopolysaccharide-induced NO production by inhibiting p38 MAPK, JNK and NF-κB activation in murine peritoneal macrophages. *Inter. Immunopharmacol.* **2007**, *7*, 1825−1833.

(29) Erdmann, K.; Grosser, N.; Schipporeit, K.; Schröder, H. The ACE inhibitory dipeptide Met-Tyr diminishes free radical formation in human endothelial cells via induction of heme oxygenase-1 and ferritin. *J. Nutr.* **2006**, *136*, 2148−2152.

Received for review June 2, 2010. Revised manuscript received July 28, 2010. Accepted September 20, 2010. This research was supported by grants from the Alberta Livestock Industry Development Fund (now Alberta Livestock and Meat Agency, ALMA), the Alberta Egg Producers (AEP), and the Natural Sciences and Engineering Research Council of Canada (NSERC) to J.W. S.T.D. is an Alberta Heritage Foundation for Medical Research (AHFMR) Scientist and a Tier 1 Canada Research Chair (CRC) in Women's Cardiovascular Health. W.H. is supported by ALMA. S.C. is supported by postdoctoral fellowships from AHFMR and the Heart and Stroke Foundation (HSF) of Canada.