Normal IgG Downregulates the Intracellular Superoxide Level and Attenuates Migration and Permeability in Human Aortic Endothelial Cells Isolated From a Hypertensive Patient

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Abstract—The normal IgG, a circulating antibody, is maintained at a constant level in humans. However, little is known regarding whether normal IgG has effects on the function of vascular endothelial cells. The purpose of this study was to investigate whether IgG affects superoxide (O$_2^-$) generation and cell permeability in human aortic endothelial cells (HAECs) isolated from a hypertensive patient. The effect of normal human IgG on endothelial cell function was investigated in cultured HAECs isolated from a hypertensive patient who died of stroke. The results demonstrated, for the first time, that normal IgG attenuated the intracellular O$_2^-$ level and decreased cell migration, cell permeability, and stress fiber formation in HAECs. IgG significantly decreased Rac1 activity and NADPH oxidase activity but upregulated Mn superoxide dismutase expression in HAECs, which may contribute to the IgG-induced decrease in O$_2^-$ level. It is noted that AMP-activated protein kinase (AMPK) was activated by IgG, as evidenced by increased phosphorylation of AMPK. Interestingly, inhibition of AMPK by an AMPK inhibitor abolished IgG-induced decreases in Rac1 and NADPH oxidase activities and IgG-induced increases in Mn superoxide dismutase expression, suggesting that AMPK is an important mediator of the IgG-induced regulation of these enzymes. Importantly, inhibition of AMPK activity also prevented the IgG-induced decrease in O$_2^-$ levels, cell migration, cell permeability, and stress fiber formation. Therefore, normal human IgG may protect HAECs via activation of AMPK and subsequent decreases in intracellular O$_2^-$.

These findings reveal a previously unidentified role of normal IgG in regulating AMPK and endothelial cell function. (Hypertension. 2012;60:818-826.) ● Online Data Supplement

Key Words: IgG • superoxide • AMPK • NADPH oxidase • endothelial cell • permeability • migration

The endothelium is a semipermeable barrier that regulates fluid and solute exchange between blood and interstitial space. Endothelial dysfunction is characterized by increased superoxide (O$_2^-$) level, impaired NO activity, increased endothelial apoptosis, and an increase in endothelial permeability. An abnormal increase in vascular permeability has been found in several pathological conditions and diseases, such as atherosclerosis, hypertension, diabetic vasculopathy, and heart failure.

The NADPH oxidase is the major source of O$_2^-$ in vascular endothelial cells. The NADPH oxidase and its regulatory subunit Rac1 (a small GTPase) play important roles in O$_2^-$ production. Phosphorylation of Rac1 increases NADPH oxidase activity and O$_2^-$ generation. O$_2^-$ impairs vascular endothelial function by increasing endothelial cell permeability and cell migration and by impairing cell-cell adhesion. Inhibition of Rac1 and NADPH oxidase shows protection in the endothelial cell barrier.

Normal human IgG purified from healthy donors has increasingly been used for the treatment of autoimmune and systemic inflammatory diseases, in addition to supportive therapy of immunodeficient patients. Previous studies showed that normal human IgG prevents endothelial cell activation induced by tumor necrosis factor-α and oxidized low-density lipoprotein atherogenic stimuli. In humans, the circulating normal IgG is maintained at a constant level. However, little is known regarding whether normal IgG has any effects on the function of vascular endothelial cells. Whether IgG affects NADPH oxidase activity and the intracellular O$_2^-$ level in human vascular endothelial cells is an interesting topic but remains poorly explored.

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that serves as an energy sensor in the regulation of cellular metabolism. Recent studies showed that AMPK is expressed in vascular endothelial cells and that AMPK activation improves endothelial function by suppressing oxidative stress. The major isoform of AMPK in endothelial cells is AMPKα1β1γ1, with α1 being the catalytic subunit. AMPK plays an important role in endothelial cells to maintain an anti-inflammatory and antiatherogenic
AMPK protects endothelial cells from various cellular stresses, including hypoxia and oxidative stress, as well as excessive exposure to free fatty acids and glucose. It is not known, however, if there exists any functional relationship between normal IgG and AMPK in human aortic endothelial cells (HAECs). The purpose of this study was to investigate a new hypothesis that IgG activates AMPK, leading to a decrease in intracellular $\text{O}_2^-$ and cell permeability in HAECs.

### Materials and Methods

See the Methods section in the online-only Data Supplement for additional details.

#### Endothelial Cell Culture

Isolated HAECs and culture medium were purchased from Life Line Cell Technology (Walkersville, MD). HAECs were isolated from a hypertensive patient who died of stroke. For the detailed culture procedure, refer to the online-only Data Supplement.

#### Reagents and Antibodies

The reagent and antibody information is provided in the online-only Data Supplement.

#### Preparation of Cell Lysates and Western Blotting

HAECs treated with normal human IgG were harvested using a cell scraper, as detailed in the online-only Data Supplement. The Western blot procedure was performed as we described recently.

#### Measurement of NADPH Oxidase Activity

NADPH oxidase activity was measured using the lucigenin chemiluminescence method, as described in our previous study. Briefly, the cells were harvested, counted, and washed twice in cold PBS. Cell pellet was resuspended with lucigenin-PBS, and the basal level was measured after incubation in the dark for 20 minutes. NADPH was added into the wells and measured in 2-minute intervals for 14 minutes.

#### Quantification of Intracellular $\text{O}_2^-$ by Dihydroethidium Using Flow Cytometry

The intracellular $\text{O}_2^-$ level was evaluated by flow cytometry based on dihydroethidium staining (red fluorescence), as we described recently. For details, refer to the online-only Data Supplement.

#### Analysis of Intracellular NO and Endothelial NO Synthase Activity by 4,5-Diaminofluorescein Fluorescence

Intracellular NO was measured in real time using NO-specific fluorescence probe 4,5-Diaminofluorescein diacetate as we described previously. The detailed procedure can be found in the online-only Data Supplement.

#### Immunofluorescence Confocal Microscopy

HAECs grown on slide chambers were treated with normal human IgG and then incubated with primary antibodies (phosphorylated AMPK and vascular endothelial [VE] cadherin) and stained with Alexa-647 Fluor secondary antibodies for confocal microscopy analysis. The detailed procedure can be found in the online-only Data Supplement.

#### Endothelial Cell Migration Assay

Endothelial cell migration was assayed using a 2D cell migration assay kit according to the manufacturer’s manual. For detailed procedures, refer to the online-only Data Supplement.

#### Permeability Assay

The cell permeability was assessed using the fluorescein isothiocyanate-dextran leaking method and detailed in the online-only Data Supplement.
Statistical Analysis
Data were analyzed by 1-way ANOVA or t test. The Tukey procedure was used to assess the significance of difference between means. Significance was set at a 95% confidence limit.

Results
IgG Dose-Dependently Decreased the Intracellular O2·− Level in HAECs
The intracellular O2·− was first evaluated using dihydroethidium staining by confocal microscopy. Normal human IgG (10 mg/mL) obviously decreased the O2·− level in HAECs from a hypertensive patient (Figures 1A and S1, available in the online-only Data Supplement). For the dose-response test, HAECs were treated with IgG at 0.5, 5.0, 10.0, and 15.0 mg/mL, respectively. The intracellular O2·− level (dihydroethidium staining) was measured using flow cytometry. IgG decreased the intracellular O2·− level in a dose-dependent manner (Figure 1B). The dose of 10 mg/mL was chosen as an optimal dose for the subsequent tests, because it is effective and does not cause obvious morphological changes (Figures 1B and S2). For the time course, HAECs were incubated with IgG (10 mg/mL) for 24, 48, and 72 hours, respectively. As shown in Figure 1C, IgG decreased the intracellular O2·− level in all 3 of the incubation times. IgG did not further decrease the intracellular O2·− level but caused slight morphological changes at a higher dose (15 mg/mL), which was not used for the remainder of the study (Figure S2). Albumin did not decrease intracellular O2·− as IgG did (Figure S3), suggesting that the suppressing effect of IgG on O2·− production and NADPH oxidase activity is IgG specific.

The basal level of intracellular O2·− in HAECs was significantly higher in a hypertensive patient than in a normal human subject (Figure S1A and S1B). Normal human IgG did not affect intracellular O2·− in HAECs from a normal human subject, although it significantly attenuated intracellular O2·− in HAECs from a hypertensive patient (Figure S1A and S1B). At the dose of 10 mg/mL, IgG decreased the O2·− in HAECs in a hypertensive patient to the control level (Figure S1A and S1B).

IgG Upregulated AMPK Activity But Downregulated NADPH Oxidase Activity
IgG significantly increased AMPK activity, as evidenced by the increased phosphorylation of AMPKα1 in HAECs (Figure 2A). However, the total AMPKα1 protein expression was not altered by IgG (Figure 2B). IgG significantly decreased NADPH oxidase activity measured using the lucigenin chemiluminescence method (Figure 2C). The intracellular level of NO or endothelial NO synthase (eNOS) activity was not altered significantly by IgG (Figure 2D).

Figure 2. IgG upregulated AMP-activated protein kinase (AMPK) activity but downregulated NADPH oxidase activity. A, Western blot analysis of phosphorylated (p-)AMPKα1. B, Western blot analysis of AMPKα1 expression. C, NADPH oxidase activity (arrows indicating addition of NADPH). RLU indicates relative light unit. D, Intracellular NO measured by 4,5-Diaminofluorescein diacetate (DAF-2 DA) fluorescence using flow cytometry. n=3 independent experiments. *P<0.05; **P<0.01; ***P<0.001.
subject (Figure S1C). IgG did not affect NADPH oxidase activity in HAECs from a normal human subject, but it significantly attenuated NADPH oxidase activity in HAECs from a hypertensive subject (Figure S1C). IgG (10 mg/mL) decreased NADPH oxidase activity in HAECs from a hypertensive patient to the control level (Figure S1C).

IgG Decreased the Intracellular $O_2^−$ Level via Activation of AMPK

To determine whether AMPK activation mediates the IgG-induced decrease in $O_2^−$ level in HAECs, we used an AMPK inhibitor (AI; compound C) to inhibit AMPK activity (phosphorylation of AMPKα1). The phosphorylated AMPKα1 indicates AMPK activity, because AMPKα1 is the catalytic subunit of AMPK in endothelial cells. AI effectively inhibited AMPK activity and abolished IgG-induced activation of AMPK (Figure 3A). Importantly, AI blocked the IgG-induced decrease in the intracellular $O_2^−$ level (Figure 3B). IgG or AI did not affect cell cycle, cell proliferation, or apoptosis in HAECs (Figure S4 and S5). IgG receptor (CD32) was not expressed in HAECs (Figure S6).

IgG Decreased Rac1 Activity via Activation of AMPK

IgG significantly decreased Rac1 activity (phosphorylated Rac1), but the AI increased Rac1 activity (Figure 4A).

Interestingly, inhibition of AMPK blocked the IgG-induced decrease in Rac1 activity, suggesting that AMPK is a key mediator in this process (Figure 4A). IgG or AI did not affect total Rac1 protein expression (Figure 4B). Rac1 is an important regulator for NADPH oxidase activity. Consistently, IgG decreased NADPH oxidase activity significantly (Figure 4C). The IgG-induced decrease in NADPH oxidase activity was abolished by AI (Figure 4C).

NOX1 protein expression was not altered significantly by treatments with IgG or AI (Figure S7A). NOX2 protein was not detectable in HAECs (data not shown). IgG did not alter...
p47phox and p67phox protein expression significantly in HAECs, although a slight decrease in these protein expression was found in the IgG-treated group (Figure S7B and S7C). Inhibition of AMPK did not affect p47phox and p67phox protein expression.

IgG Increased Mn Superoxide Dismutase Expression via AMPK
Mn superoxide dismutase (SOD) and eNOS protein expression were measured in HAECs treated with IgG and/or AI. IgG significantly increased MnSOD protein expression (Figure 5A). Inhibition of AMPK blocked the IgG-induced increase in MnSOD expression, indicating that this effect of IgG is mediated by AMPK activation. In contrast, eNOS protein expression was not affected by IgG or AI in HAECs (Figure 5B).

IgG Decreased Endothelial Cell Migration via AMPK
The effect of IgG on cell migration was assessed at 2, 5, 10, and 20 hours after treatment with IgG. IgG significantly decreased the HAEC migration at all of these time points (Figure 6). AI promoted HAEC migration. Moreover, AI blocked the IgG-induced decrease in cell migration (Figure 6). The most effective time point was 10 hours after treatment with IgG, as evidenced by greater migration of HAEC cells (Figure 6D).

IgG Decreased Endothelial Cell Permeability via AMPK
Dextran leakage was reduced significantly in IgG-treated HAECs (Figure 7A and 7B), indicating that IgG decreased cell permeability. Dextran leakage of the IgG-treated cells was ~50% of that of the untreated cells. The most effective time point was 15 minutes (Figure 7A and 7B). The IgG-induced decrease in cell permeability was abolished by AI, indicating that this effect of IgG was mediated by AMPK.

The F-actin level was decreased significantly in IgG-treated HAECs (Figure 7C and 7D), indicating that IgG inhibited stress fiber formation. This inhibitory effect was abolished by AI (Figure 7C and 7D), indicating that AMPK played an important role in IgG-induced decreases in stress fiber formation.

IgG Increased VE Cadherin Expression not via AMPK
Western blot analysis showed that IgG increased protein expression of VE cadherin (Figure 8A). VE cadherin is essential for maintaining cell-cell adhesion and endothelium permeability. However, AI failed to block the IgG-induced increase in VE cadherin expression (Figure 8A), indicating that this effect of IgG is independent of AMPK. The immunocytochemical analysis showed that IgG increased in VE cadherin expression mainly in the cell membrane (Figure 8B and 8C, single arrow) and cell-cell junction areas (Figure 8B and 8C, double arrows).

Discussion
The vascular endothelium is an important barrier that regulates the passage of macromolecules and circulating cells from blood to tissues. The endothelium is the major target of oxidant stress that is associated with several vascular disorders. Specifically, oxidant stress increases vascular endothelial permeability.31 Dysregulation of vascular permeability causes endothelial dysfunction and is observed in several life-threatening conditions, including hypertension, heart diseases, cancer, stroke, and diabetes mellitus.10 Normal IgG constitutively circulates in the human vascular system. The present investigation demonstrated, for the first time, that normal human IgG downregulated the intracellular O2·− level and decreased the permeability of HAECs isolated from a hypertensive patient (Figures 1 and 7). The suppressing effect of IgG on O2·− production is IgG specific, because another protein albumin did not affect O2·− levels (Figure S3). Although normal human IgG from healthy donors has been used for immune deficiency disorders,32–34 this is the first study suggesting that normal human IgG may have therapeutic potential for endothelial cell dysfunction incurred by oxidative stress.
Normal IgG is maintained at a constant level in the circulation. It is not clear, however, if IgG plays a role in the regulation of vascular endothelial cell function. The present study revealed that normal IgG may have beneficial effects in endothelial cells isolated from a hypertensive patient. Under pathological conditions, T cells are activated that stimulate B cells to generate diseased antibodies in response to stimulations (stress, specific antigens, angiotensin II, etc). These diseased antibodies participate in the inflammatory process and oxidative stress. Thus, normal IgG and diseased IgG have opposite functions in the cardiovascular system. It has been reported that activation of T cells may be involved in the pathogenesis of hypertension. The beneficial effect of normal IgG appears to be attributed to activation of AMPK, because inhibition of AMPK blocked the IgG-induced decreases in intracellular O$_2^-$ and cell permeability in HAECs. This result reveals a previously unidentified role of IgG in regulating AMPK activity in endothelial cells.

The IgG-induced decrease in intracellular O$_2^-$ may be attributed to the decreased NADPH oxidase activity and the increased MnSOD expression. The IgG receptor (Fc γ-receptor II-CD32) was not expressed in HAECs (Figure S6), excluding the involvement of the Fc γ-receptor in the beneficial effects of IgG. Interestingly, the IgG-induced regulation of these enzymes appeared to be mediated by the upregulation of AMPK activity, because it can be abolished by inhibition of AMPK. This finding supports a notion that AMPK is an energy sensor that regulates cellular oxidative status and cellular metabolism for maintaining normal cell function. In human and mouse endothelial cells, the AMPK activity is regulated by CaMKK-$\beta$ or LKB1. However, IgG-induced activation of AMPK may not be mediated by calcu/calmodulin-dependent protein kinase kinase $\beta$ or liver kinase B1, because IgG did not affect these upstream regulators of AMPK (Figure S8). Further studies are needed to investigate the mechanism that mediates the IgG-induced activation of AMPK.

Figure 6. IgG attenuated human aortic endothelial cells (HAEC) migration via AMP-activated protein kinase (AMPK). HAECs were treated with IgG for 24 hours. The bio-gels were then removed and allowed the cells to migrate for 20 hours. A, Photomicrographs of HAEC migration were taken before removal of bio-gel (0 hour) and at 2, 5, 10, and 20 hours after removal of bio-gel, respectively. B, Percentage of migration at 2 hours. C, Percentage of migration at 5 hours. D, Percentage of migration at 10 hours. E, Percentage of migration at 20 hours. n=3 independent experiments. *$P<0.05$; **$P<0.01$. AI indicates AMPK inhibitor. Phase contrast micrographs, $\times$100.
In endothelial cells, microtubules are cross-linked to actin filaments and can affect endothelial permeability through the effects on actin filaments. Dynamic rearrangements of microtubules affect the organization of other cytoskeletal components, and stabilization of microtubules is shown to protect endothelium against actin stress fiber formation and hyperpermeability. Depolymerization of microtubules activates guanine nucleotide exchange factors and signals through Rho family GTPases, such as Rac1, leading to actin stress fiber formation. The present data showed that normal human IgG downregulated Rac1 activity and decreased F-actin formation (Figures 4 and 7). These beneficial effects of IgG may be mediated by activation of AMPK, which can be blocked by inhibition of AMPK.

The endothelial cell dysfunction was also characterized by the increased endothelial cell migration. This motile process is directionally regulated by chemotactic and mechanotactic stimuli and further involves degradation of the extracellular matrix to enable progression of the migrating cells. O$_2^-$ increased migration of aortic smooth muscle cells. The reactive oxygen species production via the rac1-dependent NADPH oxidase stimulates diverse redox signaling pathways, leading to an increase in endothelial cell migration. A decrease in F-actin attenuates endothelial cell migration. Thus, normal human IgG decreased HAEC migration, which may be, in part, attributed to the downregulation of Rac1 and NADPH oxidase activity and the attenuation of F-actin formation (Figures 4, 6, and 7). However, a further study is required to determine whether and to what degree the inhibiting effect of IgG on endothelial cell migration is mediated by O$_2^-$ using an O$_2^-$ scavenger. AMPK, an upstream regulator of NADPH oxidase activity, may be involved in the IgG-induced decrease in HAEC migration, which can be partially blocked by inhibition of AMPK (Figure 6).

VE cadherin is important for maintaining normal endothelial monolayer permeability. Interestingly, IgG increased VE cadherin protein expression. It was reported that Rac1-induced reactive oxygen species disrupt VE cadherin–based

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**Figure 7.** IgG decreased cell permeability and stress fiber formation via AMP-activated protein kinase (AMPK). **A,** The time course of fluorescein isothiocyanate (FITC)-dextran leakage (arrow shows obvious change of FITC-dextran leakage at 15-minute time point; RFUs indicates relative fluorescence units). **B,** Cell permeability assessed by percentage change in FITC-dextran leakage at the 15-minute time point. **C,** Representative photomicrographs of stress fiber formation (F-actin, green fluorescence). **D,** Quantification of F-actin formation. n=3 independent experiments. *P<0.05, **P<0.01. AI indicates AMPK inhibitor. Magnification, ×630. Scale bar, 50 μm.
Figure 8. IgG upregulated vascular endothelial (VE) cadherin expression not through AMP-activated protein kinase (AMPK). A, Western blot analysis of VE cadherin protein expression. B, In situ expression of VE cadherin (red, arrows) by immunocytochemistry and immunofluorescence. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). VE cadherin was mainly localized in the cell membrane (single arrow) and the cell-cell junction area (double arrows). C, Quantification of VE cadherin expression based on VE-cadherin fluorescence intensity. VE-C indicates VE cadherin. Magnification, ×630. Scale bar, 50 μm.

cell-cell adhesion leading to hyperpermeability in the endothelium. Unexpectedly, inhibition of AMPK completely blocked the IgG-induced decreases in Rac1 activity and intracellular $O_2^\cdot$ levels but failed to block the IgG-induced increase in VE cadherin expression. This result suggests that the IgG-induced increase in VE cadherin expression may not be mediated by the AMPK-Rac1/NADPH oxidase pathway, although the underlying mechanism remains to be found.

AMPK activation was reported to reduce hyperglycemia-induced mitochondrial reactive oxygen species production by induction of the MnSOD pathway in endothelial cells. Silencing of AMPKα1 in human endothelial cells decreases the expression of several antioxidant defense-related genes, including MnSOD, leading to accumulation of reactive oxygen species. Interestingly, IgG increased MnSOD expression in HAECs (Figure 5), which may contribute to the IgG-induced decrease in intracellular $O_2^\cdot$. The IgG-induced upregulation of MnSOD expression was likely mediated by activation of AMPK, because it can be blocked by inhibition of AMPK. In contrast, IgG did not affect the intracellular NO level (Figure 2), eNOS activity, or eNOS protein expression (Figure 5).

**Perspectives**

NADPH oxidase activity and $O_2^\cdot$ production are increased in aortic endothelial cells isolated from a hypertensive patient. It is interesting and significant that normal human IgG down-regulated the intracellular $O_2^\cdot$ level and decreased cell migration, cell permeability, and stress fiber formation in HAECs. The beneficial effects of IgG may be mediated by activation of the AMPK, which regulates several downstream factors, such as Rac1, NADPH oxidases, and MnSOD, leading to endothelial protection. These novel findings suggest that normal human IgG may be a potential pharmacological candidate for the treatment of endothelial dysfunction. Future studies are warranted to test this hypothesis in vivo.

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**Disclosures**

None.

**References**

Novelty and Significance

What Is New?

- It is new and interesting that normal human IgG attenuates intracellular O$_2^-$ production and protects endothelial cells by decreasing cell permeability, migration, and stress fiber formation.
- This is the first report that normal human IgG activates endothelial AMPK and regulates endothelial cell function.

What Is Relevant?

- It is significant that normal IgG protects endothelial cells isolated from a hypertensive patient.
- IgG holds a promise for treating endothelial dysfunction associated with hypertension and diabetes mellitus.

The finding has important translational potential because normal human IgG can be readily used for testing its protective effects on endothelial cells in hypertensive patients.

Summary

Normal human IgG may protect HAEcs via activation of AMPK and subsequent decreases in intracellular O$_2^-$ production. These findings reveal a previously unidentified role of IgG in regulating AMPK and endothelial cell function.
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