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Loss of menin mediated by endothelial cells treated with CoPP is associated with increased maturation of adipocytes

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Abbreviations: HO-1/HO-2, heme oxygenase-1,2; *Men1*, multiple endocrine neoplasia type 1; hsp, heat shock proteins; CoPP, cobalt protoporphyrin IX; Ang II, angiotensin II; CM, conditioned media; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor; IL-6, interleukin 6; MCP-1, monocyte chemotactic protein-1

Oxidative stress is caused by an increase in reactive oxygen species (ROS) relative to the antioxidant defense system. An increase in ROS is known to decrease vascular function, increase inflammatory cytokines, and promote adipocyte hypertrophy. A known regulator of the oxidative stress response is the heat shock protein, heme-oxygenase 1 (HO-1), which is induced by cobalt protoporphyrin IX (CoPP). Menin was recently found to promote the sustained expression of heat shock proteins and is implicated in the regulation of oxidative stress. In this study, we investigated how changes in menin expression affected adipogenesis via the interaction between endothelial cells and adipocytes in response to CoPP treatment during oxidative stress. Using angiotensin II (Ang II) to induce oxidative stress in endothelial cells and adipocytes, we observed the induction of various cytokines including EGF, VEGF, angiogenin, IL-6, and MCP-1. Preadipocytes cultured in endothelial cell conditioned media treated with Ang II showed no changes in differentiation markers. Preadipocytes treated with the endothelial cell-conditioned media pretreated with CoPP resulted in an increase in the number of adipocytes, which expressed higher levels of adipocyte differentiation markers in direct correlation with the complete downregulation of the stress response regulator, menin. This change was not detected in adipocytes directly treated with CoPP alone. Therefore, we concluded that loss of menin is associated with the maturation of adipocytes induced by conditioned media from endothelial cells treated with CoPP.

Introduction

Oxidative stress is caused by overproduction of reactive oxygen species (ROS) and accompanies various other diseases including diabetes, endothelial dysfunction, and cardiovascular disease.[1,2] Physiologically, ROS play an important role in cellular signaling, apoptosis, autophagy, and programmed necrosis.[1,3] In pathology, there is an accumulation of ROS resulting in cellular damage, increased inflammatory cytokines, and in the endothelium, increased adhesion molecules.[1,4]

A major inducer of oxidative stress in endothelial dysfunction is angiotensin II (Ang II).[5] Ang II is a vasoconstrictor that plays an important role in the renin–angiotensin system (RAS).[5-7] In an environment of oxidative stress, there is an excessive amount of Ang II, which can activate the PI3K/AKT kinase pathway and eNOS to further produce ROS, leading to endothelial dysfunction and hypertension.[6,8] Both adipose tissue and endothelial cells produce Ang II and when stimulated by oxidative stress, produce excessive amounts of Ang II leading to a pathological state.[9]

The defense mechanism against oxidative stress and ROS is the upregulation of antioxidants, specifically heme-oxygenase 1 (HO-1).[10-12] When HO-1 increases, anti-oxidant and anti-inflammatory molecules increase and the level of ROS decreases.[13,14] Other benefits of increased levels of HO-1 protein include the prevention of high blood pressure, decreased vasoconstrictors (Ang II and ET-1), and increased vasodilators (CO and NO).[14-18] HO-1, also known as a heat shock protein, is regulated by a plethora of signaling molecules involved in oxidative stress response, including the MAPK and AKT pathways.[19,20]

A known stress and heat shock protein regulator, menin, is the 67-kDa protein product of the multiple endocrine neoplasia 1 (MEN1) tumor suppressor gene.[21,22] MEN1 patients have a higher prevalence of type 2 diabetes, risk of cardiovascular disease, and premature cardiovascular death.[23,24] Recently, it was shown that menin is required for the sustained expression of heat shock proteins in response to hypoxia, and oxidative stress.[22,25] Menin is also known to inhibit the stress response pathway of the MAPK cascade.[26,27]

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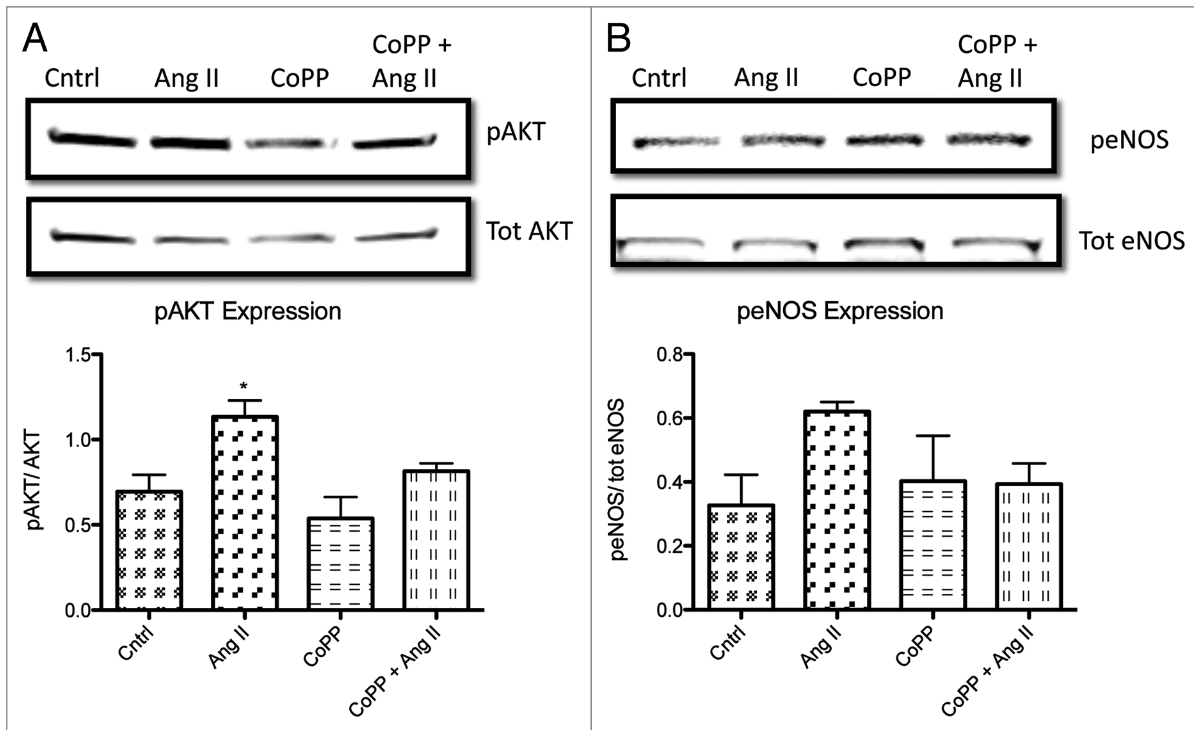

Figure 1. CoPP protects against the angiotensin II induced oxidative stress in endothelial cells. Endothelial cells were treated overnight with 20 μM Ang II alone (Ang II), 2.5 d with 2 μM CoPP alone (CoPP) or pretreated with 2.5 d with 2 μM CoPP, then treated overnight with 20 μM Ang II (CoPP + Ang II). (**A**) Western blot showing an increase in pAKT protein expression when treated with Ang II that is reversed with CoPP treatment. $^*P < 0.05$ vs. control, $n > 3$. (**B**) Representative western blot showing an increase in peNOS when treated with Ang II that is reversed with CoPP treatment, $n > 3$.

In this study, we will investigate whether changes in menin expression mediate the HO-1 regulation of oxidative stress through the interaction of endothelial cells treated with the HO-1 inducer, cobalt protoporphyrin IX (CoPP), on pre-adipocytes to promote the formation of healthy and mature adipocytes.

Results

Ang II induced oxidative stress in endothelial cells. Ang II is one of the major factors that causes an over production of ROS in obesity and endothelial dysfunction.[5] Both endothelial cells and adipocytes produce excessive amount of Ang II in an environment of oxidative stress, further causing a significant increase in ROS.[28-31] We replicated this pathological environment by using 20 μM of Ang II to treat endothelial cells. Consistent with literature, we saw Ang II produced oxidative stress as measured by an increase in pAKT and peNOS levels, known to increase superoxide production (**Fig. 1A and B**, respectively).

To further prove that Ang II induced oxidative stress in the endothelial cells, we measured various cytokines secreted by the endothelial cell in the conditioned media. We observed changes in the cytokines IL-6 and MCP-1, and the angiogenesis factors vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and angiogenin. VEGF is necessary for proper stress response in endothelial cells and is elevated in oxidative stress in response to hypoxia.[32] EGF is increased in oxidative stress and in high amounts, exacerbates the production of oxidative stress.[33] Finally, angiogenin is a potent stimulator of new blood vessels and is decreased with oxidative stress.[34] Consistent with the literature, we observed a significant increase in the inflammatory cytokines, MCP-1 and IL-6 (**Fig. 2A and B**) as well as the angiogenesis factors, VEGF and EGF (**Fig. 2C and D**). Furthermore, we detected a decrease in angiogenin (**Fig. 2E**). As expected, Ang II was significantly higher in the media treated with Ang II (**Fig. 2F**). Together, these results indicate that the treatment of Ang II induced oxidative stress in endothelial cells by upregulating inflammatory cytokines and downregulating angiogenesis factors.

CoPP protects endothelial cells from oxidative damage. Cobalt protoporphyrin IX (CoPP) is a potent HO-1 inducer. HO-1 plays a protective role in endothelial cells; however, the mechanisms are not well known. We hypothesized that increasing antioxidants, specifically HO-1, would protect endothelial cells from the effects of Ang II. In **Figure 3A**, we observed a significant increase in HO-1 with treatment of CoPP alone or pretreatment of CoPP followed by Ang II (CoPP + Ang II). We determined that an increase in HO-1 via CoPP reversed the effect of Ang II on pAKT and peNOS levels, thus protecting the endothelial cells from the effects of Ang II (**Fig. 1A and B**).

Furthermore, we examined the effects of CoPP treatment on the cytokine expression in the endothelial cell conditioned media. We observed no protective effect of CoPP on the expression of the inflammatory cytokines MCP-1 and IL-6 or the angiogenesis factors VEGF and EGF induced by Ang II (**Fig. 2**). While CoPP alone did not induce the secretion of cytokines by the endothelial

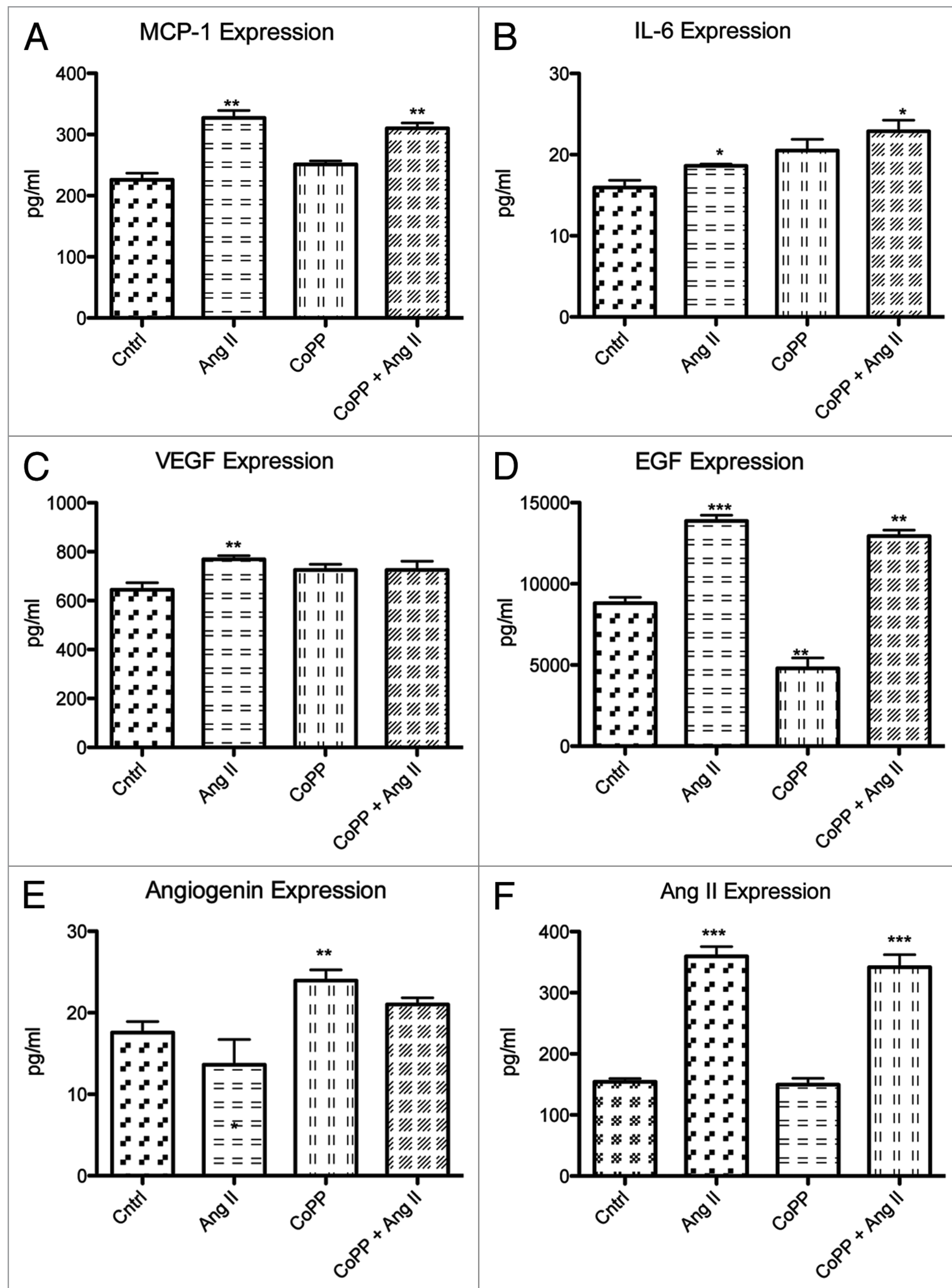

Figure 2. Ang II induces inflammatory cytokines in endothelial cell conditioned media. Conditioned media from the endothelial cells was collected after the respective treatments and cytokines were measured by an ELISA. $n = 5$ unless otherwise noted. (**A**) MCP-1 expression is increased with Ang II and CoPP + Ang II. $^{**}P < 0.005$ vs. control. (**B**) IL-6 expression is increased with Ang II and CoPP + Ang II treatment. $^{*}P < 0.05$ vs. control. (**C**) VEGF expression is significantly increased with treatment of Ang II but is not changed with treatment of CoPP + Ang II. $^{**}P < 0.005$ vs. control. (**D**) EGF expression is increased with treatment of Ang II and CoPP + Ang II; however, it is significantly decreased with treatment of CoPP. $^{***}P < 0.005$ vs. control, $^{**}P < 0.005$ vs. control. (**E**) Angiogenin expression is increased with CoPP treatment. $^{**}P < 0.005$ vs. control. (**F**) Angiotensin II expression is significantly increased in both the Ang II and CoPP + Ang II treatments. $^{***}P < 0.0005$ vs. control. $n = 3$.

cells, we observed a significant increase in angiogenin in the CoPP treated conditioned media confirming the involvement of HO-1 in angiogenesis (**Fig. 2E**).[32,35]

CoPP reverses the effect of Ang II on menin expression in endothelial cells. Recently, it was reported that menin can regulate as well as be regulated by heat shock proteins,[22,25] and since HO-1 is a known heat shock protein 32 (Hsp32), we hypothesized that CoPP may reverse the effects of Ang II on menin via an increase in HO-1 expression. **Figure 3A** shows the induction of HO-1 by CoPP. In **Figure 3B**, we observed a 3-fold

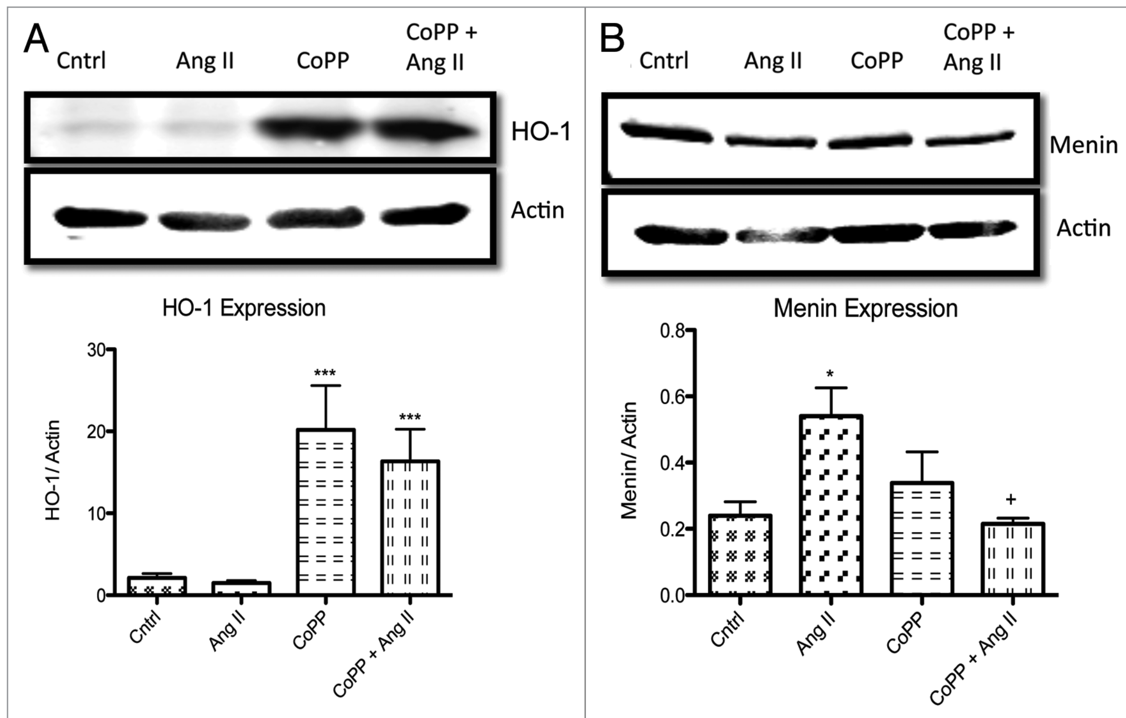

Figure 3. CoPP induces HO-1 and protects against an increase in Ang II induced menin levels in endothelial cells. (**A**) Western blot representing a significant increase in HO-1 with CoPP and CoPP + Ang II treatment. ***$P < 0.0005$ vs. control, $n > 4$. (**B**) Western blot showing a significant increase in menin expression with Ang II that is attenuated with treatment of CoPP + Ang II. *$P < 0.05$ vs. control, $^+P < 0.05$ vs. Ang II, $n > 5$.

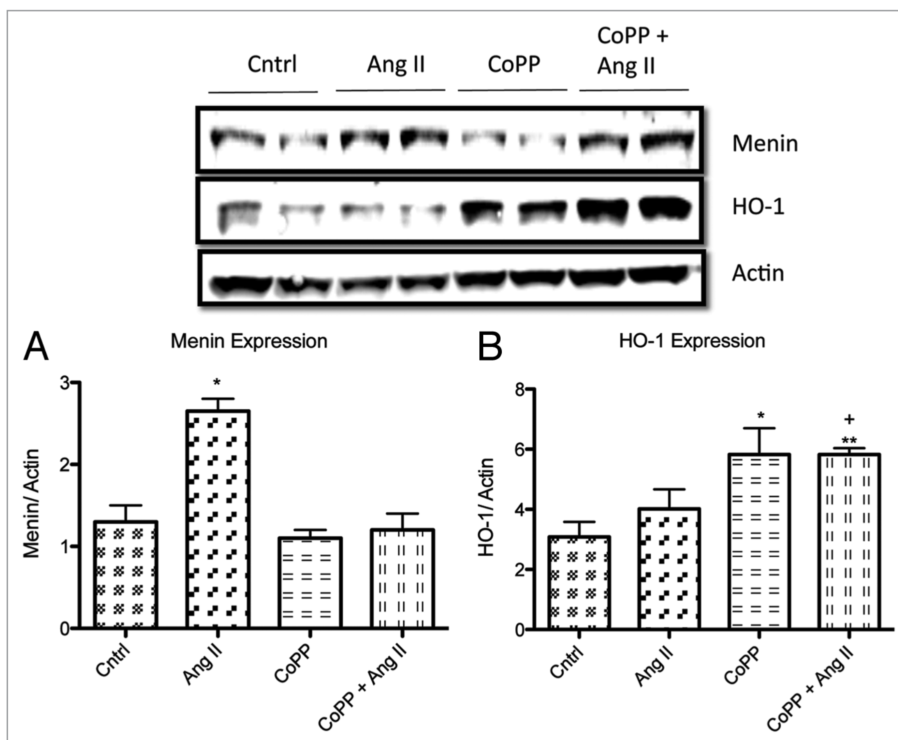

Figure 4. CoPP induced HO-1 and protected against Ang II induced menin in adipocytes. (**A**) Representative western blot showing increased menin levels with Ang II treatment. *$P < 0.05$ vs. control, $n = 3$. (**B**) HO-1 is significantly increased with CoPP treatment. *$P < 0.05$ vs. control, **$P < 0.005$, $^+P < 0.05$ vs. Ang II. $n = 5$.

increase in menin levels with treatment of Ang II that was significantly attenuated with treatment of CoPP.

In agreement with the endothelial cell data, we observed a significant increase in HO-1 in adipocytes treated with CoPP and menin when treated with Ang II (**Fig. 4**). Additionally, CoPP protected the adipocytes from the increase in menin after treatment with Ang II. We did not detect any changes in the signaling markers pAKT or peNOS (data not shown). These results illustrate that Ang II and CoPP regulate menin expression in adipocytes as well as endothelial cells.

The cytokines produced by the adipocytes were also measured. We only detected an increase in IL-6 when treated with Ang II (**Fig. 5**). Treatment with CoPP and CoPP + Ang II significantly reduced MCP-1 levels perhaps showing a protective effect against oxidative stress. However, we still observed an increase in IL-6 with CoPP + Ang II indicating that CoPP does not protect against all inflammatory cytokines. Ang II levels were increased with Ang II treatments while angiogenin levels did not change.

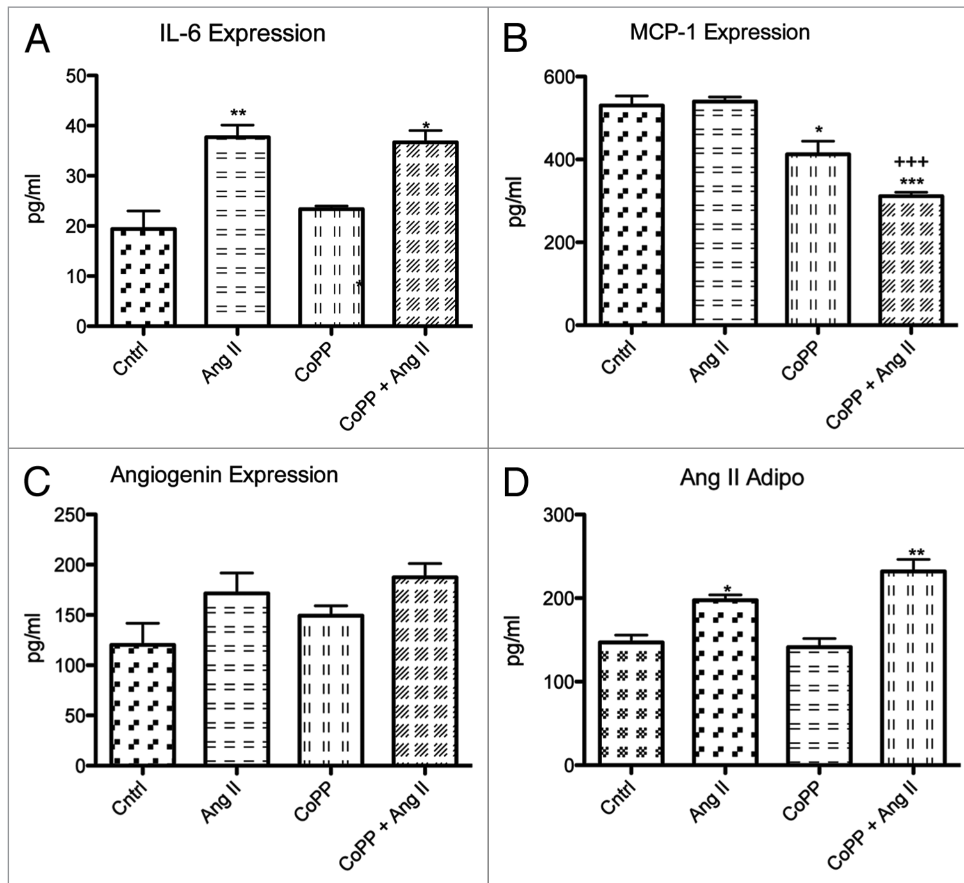

Figure 5. Ang II induced IL-6 while CoPP reduced MCP-1 levels in adipocyte conditioned media. Conditioned media was collected after respective treatments. $n = 5$ unless otherwise noted. (**A**) Treatment with Ang II and CoPP + Ang II resulted in an increase in IL-6. $^{**}P < 0.005$ vs. control $^{*}P < 0.05$ vs. control. (**B**) MCP-1 levels are significantly reduced with CoPP and CoPP + Ang II treatment. $^{*}P < 0.05$ vs. control, $^{***}P < 0.0005$ vs. control, $^{+++}P < 0.0001$ vs. Ang II. (**C**) Angiogenin levels did not change with either CoPP or Ang II treatment. (**D**) Ang II is increased with Ang II and CoPP + Ang II treatments. $^{*}P < 0.05$ vs. control, $^{**}P < 0.005$ vs. control, $n = 3$.

Endothelial cells treated with CoPP produce factors that increase adipogenesis and decrease menin in adipocytes. Adipocytes and endothelial cells produce factors that regulate each other in normal physiology and pathology; however, little is known about the mechanisms involved in this interaction.[9] We collected the conditioned media (CM) from the endothelial cells and treated pre-adipocytes with 20% of the CM for one week. We visually observed an increase in lipid droplet size in adipocytes treated with Ang II CM from endothelial cells and a decrease in lipid droplet size in the cells treated with CoPP CM (**Fig. 6**). Moreover, we determined a significant increase in the number of adipocytes of the pre-adipocytes treated with the CM from endothelial cells treated with either CoPP or CoPP + Ang II CM (**Fig.** 7). Interestingly, there was no change in expression of the signaling markers pAKT or peNOS (data not shown), indicating that pAKT and peNOS are not directly responsible for the changes in number of adipocytes associated with CoPP treatment. However, menin was completely abolished in the adipocytes treated with CoPP and CoPP + Ang II CM (**Fig. 8**) contrary to no change in menin expression observed with the direct treatment of Ang II and CoPP (**Fig.** 4). Furthermore, CoPP CM and CoPP + Ang II CM did not induce the expression of HO-1, confirming that CoPP is no longer actively present in the conditioned media of the endothelial cells (**Fig. 8**). These results demonstrate that the CoPP induced factors in endothelial cells that increased the number of adipocytes in association with decrease in menin levels.

Factors produced from endothelial cells treated with CoPP cause the maturation of adipocytes. The complete loss of menin in adipocytes treated with endothelial cell conditioned media is an entirely novel finding. Since studies have shown that menin is not expressed in mature adipose tissue,[21] we hypothesized that CM from CoPP treated endothelial cells induced the maturation of adipocytes. To determine the maturation of the adipocytes we used various mRNA markers to investigate changes in expression. Significant induction of glucose transporter type 4 (GLUT4) (**Fig. 9A**), peroxisome proliferator-activated receptor gamma (PPARγ) (**Fig. 9B**), lipoprotein lipase (LPL) (**Fig. 9C**), and fatty acid binding protein-4 (FABP4) (**Fig. 9D**) were observed in adipocytes cultured in CoPP CM or CoPP + Ang II CM compared with direct CoPP and CoPP + Ang II exposure. PPARγ plays a major role in converting preadipocytes into adipocytes while GLUT4 contributes to the metabolic function of the adipocyte by facilitating glucose transport.[36] FABP4 is a mature adipocyte

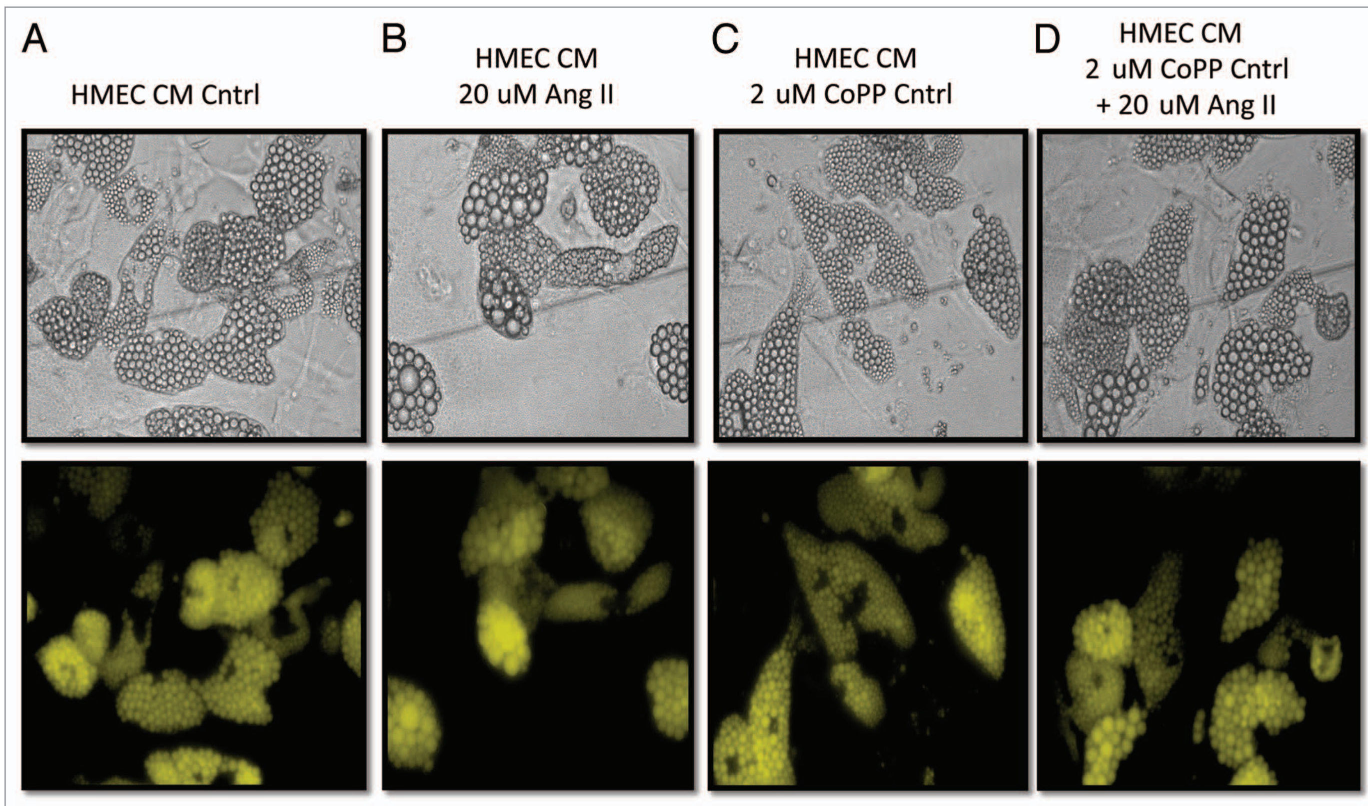

Figure 6. Lipid droplets in adipocytes treated with endothelial cell condition media (CM). Adipocytes were differentiated for 2 weeks and treated for one week with 20% endothelial cell CM. (**A**) Adipocytes treated with control endothelial cell CM. (**B**) Adipocytes cultured with endothelial cell CM with Ang II resulted in larger lipid droplets sizes. (**C**) Adipocytes treated with endothelial cell CM after CoPP treatment resulted in reduced lipid droplet sizes. (**D**) Adipocytes cultured in endothelial cell CM treated with CoPP + Ang II resulted in protected adipocytes and mixed sizes of lipid droplets.

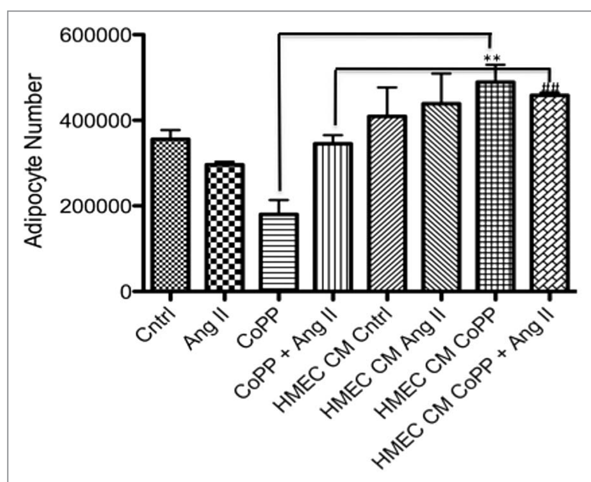

Figure 7. Endothelial cell CM treated with CoPP increased the number of adipocytes. There was an increase in the number of adipocytes with preadipocytes exposed to CM from endothelial cells treated with CoPP (HMEC CM CoPP) or CM with CoPP and Ang II (HMEC CM CoPP + Ang II) compared with CoPP or CoPP + Ang II alone.

marker known to play a role in the uptake of fatty acids[37] and LPL plays a role in the transport of lipids and is increased as adipocytes differentiate.[38,39] In accordance with our hypothesis, we observe that factors produced by CoPP in endothelial cells increased the maturation of adipocytes.

Discussion

Adipocytes and endothelial cells produce factors that regulate each other, but little is known about the mechanism of this interaction. Treatment with Ang II induced oxidative stress in both endothelial cells and adipocytes as shown by an increase in pAKT, various inflammatory cytokines, and menin. An increase in HO-1 via CoPP protected endothelial cells from an increase in pAKT and peNOS which can further exacerbate ROS production;[40,41] however, it didn't protect against an increase in the inflammatory cytokines MCP-1, IL-6, and the angiogenesis factor EGF. This indicates that induction of HO-1 by CoPP and pretreatment with CoPP before Ang II treatment does not fully protect endothelial cells from the induction of inflammatory cytokines. In adipocytes, we detected a partial protection of CoPP. We observed a mix of small and larger adipocytes when treated with CoPP + Ang II, albeit this was not quantified. Interestingly, we continued to see an increase in IL-6 but MCP-1 was significantly decreased. This may indicate an improvement in adipocyte function via a decrease in MCP-1, which would lessen the infiltration of macrophages. Further studies will be completed to determine the exact mechanisms of CoPP, Ang II,

and various cytokines in endothelial cells and adipocytes.

In this study, we have discovered that the downregulation of menin is associated with an increase in the number of mature adipocytes induced by factors secreted by endothelial cells in response to treatment of HO-1 inducer CoPP. HO-1 plays an important role in the regulation of oxidative stress in endothelial cells; however, the molecular interaction with adipocytes is not well understood. The phenomenon that endothelial cells treated with CoPP secrete factors that completely abolish menin levels and stimulate the maturation of adipocytes may provide some clues for further investigation to possible mechanisms.

Menin plays a role in numerous biological processes, including differentiation and the control of gene expression.[21,42] Recently, menin was discovered to play an important role in stress response as a regulator of heat shock proteins.[22,25] An increase in menin is initially detected during stress to recruit heat shock proteins (hsps) and eventually decreases in response to the accumulation of hsps.[22] Consistent with this finding, we observed an increase in menin when stimulated by oxidative stress and observe that when HO-1, also known as Hsp32, is overexpressed via CoPP treatment there is a decrease in menin levels.

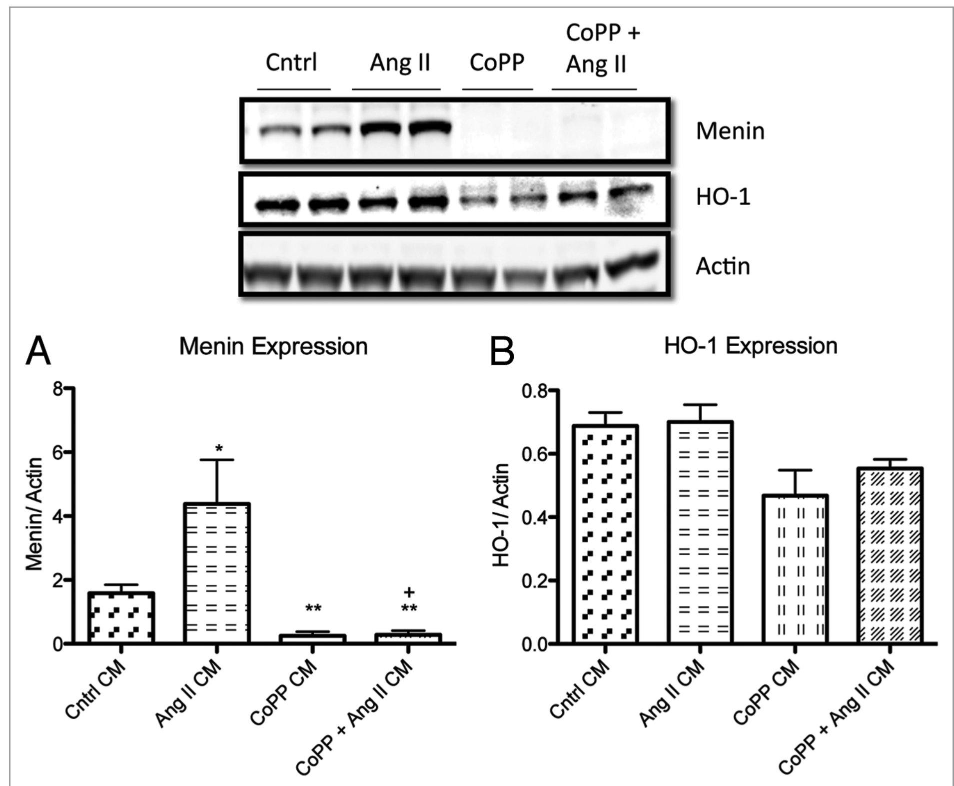

Figure 8. Adipocytes cultured with endothelial cell CM treated with CoPP abolishes menin expression. (**A**) Adipocytes treated with endothelial cell CM with Ang II resulted in a significant increase in Menin levels. Furthermore, we see complete loss of menin expression in the adipocytes cultured with CoPP and CoPP + Ang II endothelial cell CM. (**B**) HO-1 expression does not change after treatment with endothelial cell CM.

Menin causes mesenchymal stem cells to differentiate into osteoblasts and is not expressed in mature adipocytes.[21,42,43] HO-1 promotes healthy adipocytes by upregulating the adipokine, adiponectin, and increasing angiogenesis to supply vital growth factors and nutrients.[20,44,45] Indeed, when treated with CoPP to induce HO-1, we observe an increase in mature adipocyte markers, number of adipocytes, and angiogenic markers in endothelial cells in association with the complete ablation of menin levels. This indicates that CoPP treatment in endothelial cells causes the inhibition of menin in adipocytes to promote maturation. It is interesting to note that Ang II did not influence the effect of CoPP on the adipocytes.

In summary, these findings demonstrate that the HO-1 inducer CoPP stimulates endothelial cells to produce factors that act on adipocytes to facilitate an interaction associated with the complete loss of menin expression and increased adipogenesis.

Discovering new markers that modulate the regulation of ROS can aid in the discovery of a drug target to inhibit inflammatory responses in oxidative stress, known to exacerbate diseases such as type 2 diabetes, obesity, hypertension, and cardiovascular disease.

Materials and Methods

Cell culture. Human primary dermal microvascular cells (HMECs) were purchased from ATCC and maintained at 37 °C and 5% CO_2 in vascular basal medium containing 1% penicillin and streptomycin, 5 ng/mL VEGF, 5 ng/mL EGF, 5 ng/mL FGF, 15 ng/mL IGF-1, 10 mM L-glutamine, 0.75 units/mL heparin sulfate, 1 μg/mL hydrocortisone, 50 μg/mL ascorbic acid, and 2% fetal bovine serum.

Bone marrow derived mesenchymal stem cells (MSCs) were purchased from All Cells and maintained at 37 °C and 5% CO_2 in α-mem with 10% FBS, 1% penicillin and streptomycin, and 1% glutamine.

Adipocyte differentiation. MSCs were grown until 80% confluent and the media was changed to adipogenic media containing 10% FBS, 100 nM dexamethasone, 100 ug/mL insulin, 500 uM indomethacin, and 1% penicillin and streptomycin. This media was changed every 2 days for 3 weeks. MSCs were also kept in α-mem for 3 weeks as a control.

Cell treatments. HMECs were grown until 60% confluent then treated with 2 μM CoPP for 2.5 d. The media was then changed and the cells were treated with 20 μM Angiotensin II (Ang II) overnight. The conditioned media was collected and stored in –80 °C until analyzed.

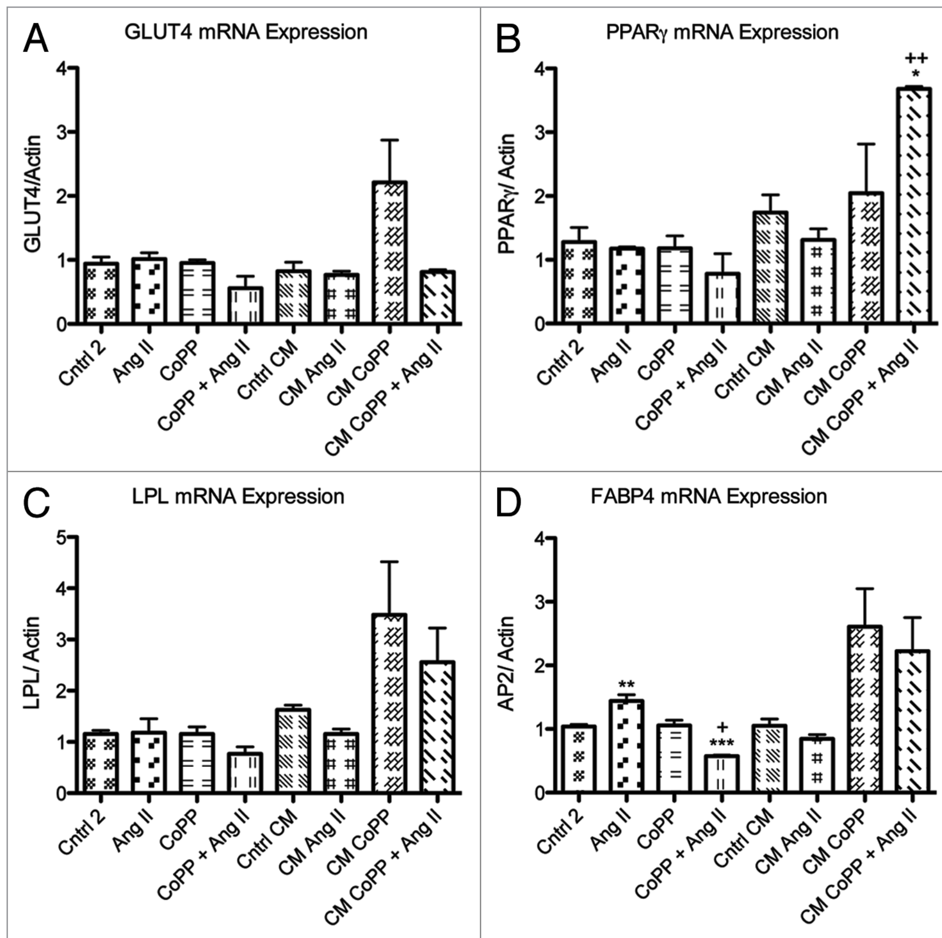

Figure 9. Endothelial cells treated with CoPP promotes the maturation of adipocytes. mRNA expression of various adipocyte markers, $n > 2$. (**A**) GLUT4 expression is increased in adipocytes treated with endothelial cell CoPP alone CM, even more than the direct treatment of CoPP. (**B–D**) PPARγ, LPL, and FABP4 expression is increased with CoPP and CoPP + Ang II CM treatment in adipocytes, more than adipocytes treated directly with CoPP and CoPP + Ang II.

Undifferentiated MSCs and differentiated adipocytes were plated in T-75 flasks for 3 weeks as stated above and then treated for 2.5 d with 2 μM CoPP. The media was changed and the cells were treated with 20 μM Ang II overnight. The conditioned media was collected and stored in −80 °C until analyzed.

Conditioned media treatments. HMECs were grown until 80% and then treated with 20% of the conditioned media from the cell treatments of undifferentiated MSCs and differentiated adipocytes described above. The undifferentiated MSCs were grown to 80% confluency and then treated with 20% of the conditioned media from the treated HMECs described above. The adipocytes were treated with the adipogenic media for 2 weeks and then with 20% conditioned media from the treated HMECs for a week.

Nile red staining. Differentiated and undifferentiated MSCs were stained with 2 mg/μl of Nile red (Acros Organics) for 15 min to stain the lipids. Cells were visualized with 100× objective lens on a Nikon Eclipse E800 microscope and photographed with a Sensys digital camera. Adipocyte number was measured using ImagePro Analyzer (MediaCybernetic, Inc.). The number of adipocytes quantified was based on the size by the area (pixels), $n = 5$.

Western blots. The cells were treated as described above and the protein was collected and immediately analyzed. The concentrations of proteins from total cell lysates were quantified using the BCA protein assay (Pierce Biotechnology, Inc.) prior to analysis on 10% TG gels and immune-probed with specific antibodies. Antibodies against rabbit-Menin (AbCam), rabbit-pAKT and rabbit-tot AKT (Cell Signaling), rabbit-HO-1 (Stressgen, Biotechnologies Corp.), menin (Bethyl Laboratories), goat-peNOS and rabbit-tot eNOS (Santa Cruz Biotechnology), and goat-Actin (Santa Cruz Biotechnology) were used. Proteins were detected by Odyssey INFRA-Red imaging system (Li-Cor Biosciences) using corresponding secondary antibodies conjugated to fluorescent dyes of different wavelengths.

Cytokine analysis. The conditioned media was collected as described above and sent to AssayGate, Inc. for analysis.

Real-time PCR. Total RNA was recovered from the adipocytes following the PerfectPure RNA Tissue Kit (5Prime) RNA extraction protocol with DNase treatment. cDNA was made using the Improm Reverse Transcriptase kit (Promega). Primers for the adipocytes were ordered from SABiosystems: LPL (PPH00023B), PPARγ (PPH2291F), FABP4 (PPH02382E), GLUT4 (PPH02326A), and Actin (PPH00073E). The thermal cycling conditions were 95 °C for 20 sec followed by 40 cycles of 95 °C for 3 min, 60 °C for 30 sec, and finally 95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 15 sec.

Statistical analysis. Data were analyzed with SPSS software using one-factor ANOVA analysis or the Student *t* test. $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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