Heme Oxygenase-Derived Carbon Monoxide Restores Vascular Function in Type 1 Diabetes

Luigi F. Rodella[3], Luca Vanella[1], Stephen J. Peterson[2], George Drummond[1], Rita Rezzani[3], John R. Falck[4] and Nader G. Abraham[1,2,*]

[1]*Departments of Pharmacology and* [2]*Medicine, New York Medical College, Valhalla, NY, USA;* [3]*Department of Biomedical Science, University of Brescia, Italy;* [4]*University of Texas Southwestern Medical Center, Dallas, USA*

Abstract: Increased heme oxygenase-1 (HO-1) expression improves vascular function by decreasing superoxide and increasing antioxidant levels. We therefore examined if HO-1 induction increased serum adiponectin levels and ameliorated vascular dysfunction in Type 1 diabetes. Administration of either carbon monoxide (CORM-3) or the HO-1 inducers, Resveratrol, and cobalt protoporphyrin (CoPP), increased serum levels of adiponectin (high molecular weight) in diabetic (streptozotocin; STZ-induced) Sprague Dawley rats. Resveratrol and CoPP administration increased HO-1 protein expression and HO activity in the aorta and significantly ($p<0.05$) increased serum adiponectin levels, compared to untreated diabetic rats. The results obtained with the CO releasing molecule, CORM-3, indicate a direct involvement of CO leading to increased levels of adiponectin. The increase in adiponectin was associated with a significant decrease in circulating endothelial cells (CEC) ($p<0.002$), decreased EC fragmentations and a significant increase in thrombomodulin (TM) and $CD31^+$ cells ($p<0.05$). Increased adiponectin levels were associated with a decrease in TNF-α-induced ICAM-1 and VCAM-1 and caspase 3 activity in endothelial cells while phosphorylation of eNOS at Ser-1179 increased. The adiponectin mediated increase in peNOS and pAKT was prevented by the phosphatidylinositol-3 kinase inhibitor, LY294002. In conclusion, there appears to be a temporal HO-1–adiponectin relationship that has a key role in vascular protection in Type 1 diabetes *via* a mechanism that involves increased levels of carbon monoxide.

Keywords: Vascular repair, diabetes, thrombomodulin, adiponectin, inflammation, carbon monoxide.

INTRODUCTION

Circulating adiponectin downregulates the generation of the classical and pro-inflammatory risk factors that mediate vascular dysfunction [1, 2]. Adiponectin, AcRP30 or adipo-*Q*, appears to play a critical role in vascular disease as increases in the levels of serum adiponectin have been associated with the amelioration of vascular dysfunction [for review see Hopkins *et al.*] [3]. Reduced adiponectin levels have been implicated in the development of obesity-linked diseases, including diabetes, vascular inflammation and cardiovascular disease [1, 3-5]. In addition, reduced plasma adiponectin levels have been reported in patients with coronary artery disease and diabetes, presumably as a result of increases in reactive oxygen species (ROS) [6-8]. Diabetes and hyperglycemia-mediated local formation of ROS are considered to be major contributing factors to endothelial cell dysfunction, leading to endothelial cell death and abnormalities in cell cycling [9-11]. Lin *et al.* described the significance of ROS production in adipocytes and the associated insulin resistance and decline in serum levels of adiponectin [12, 13] suggesting that increased levels in ROS are associated with a concomitant decrease in adiponectin. Recently, L'Abbate *et al.* reported that induction of heme oxygenase-1 (HO-1) decreased superoxide and ROS generation with a concomitant increase in adiponectin and a subsequent increased tolerance to ROS in the heart [14].

*Address correspondence to this author at the New York Medical College, Valhalla, NY 10595, USA; Tel: (914) 594-4132, Fax: (914) 594-4119; E-mail: nader_abraham@nymc.edu

Hyperglycemia-mediated cardiovascular complications and atherosclerosis contribute to the formation of O_2^- [15] and reactive oxygen species (ROS), each of which contribute to endothelial dysfunction and apoptosis [16]. It has been shown that these abnormalities are reversible with either increased expression of antioxidant enzymes or administration of antioxidant agents [17]. A reduction in antioxidant reserves has been related to endothelial cell dysfunction in diabetes, even in patients with well-controlled blood glucose levels [16, 18]. Similarly, it has been shown that moderately to well controlled type 2 diabetics demonstrate an increase in LDL oxidation [19]. Conditions associated with an elevation of oxidative stress lead to an increased level of endothelial cell sloughing [20-22]. In a recent study, diabetes was also found to be associated with increased endothelial apoptosis [9, 23].

Previously, we and others have shown that upregulation of HO-1 gene expression provides vascular protection by decreasing ROS levels and endothelial cell death [9, 24, 25]. An increase in HO-1 protein levels is associated with a parallel increase in EC-SOD and eNOS, increased activation of AKT and an increase in mitochondrial function [25-27]. The vascular and cellular protective effect of HO-1 in diabetes has been attributed to HO-1-derived carbon monoxide (CO) and bilirubin [28-31]. The absence of HO-1 exacerbates diabetes-mediated endothelial cell death [9, 24]. Thus increased expression of HO-1 protein appears to be an important defense mechanism in the maintenance of vascular integrity, as reflected by increased levels of thrombomodulin (TM) and

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CD 31; reduced levels of TM and CD31 are an indication of the development of atherosclerotic lesions [32, 33]. Reduction in plasma TM is associated with an increased risk for cardiovascular disease [25, 34]. Conversely, increased expression of TM has been shown to limit thrombus formation as well as neointimal growth [35]. Vascular dysfunction in cardiovascular disease and in diabetes can be seen in conditions that result from an increase in the levels of adhesion molecules (CAMs) and TNF-α [36-38]. Inhibition of vascular inflammation, represented by a reduction in ICAM-1 and VCAM-1 levels, contributes to an improvement in vascular function [37, 39, 40]. This can be achieved by overexpression of the HO-1 gene [9, 25, 41].

The aim of the present study was to investigate the mechanism of HO-1 vascular protection in normal and streptozotocin (STZ) induced diabetic rats. We used the HO inducers, Resveratrol and CoPP, to manipulate HO activity to study the effects of HO activity on serum adiponectin levels. The successful use of the CO donor CORM-3, indicates that CO has a direct effect on both CEC and improving vascular protection. Both CO and HO-1 mediated vascular protection are in agreement with the effect of increased levels HO-1 on the prevention of pancreatic destruction in type 1 diabetes [25, 41]. We report here that induction of HO-1 was coupled with a parallel increase in serum adiponectin levels and a concomitant decrease in endothelial cell death that occurred both *in vivo* and *in vitro* and appears to require an increase in carbon monoxide.

METHODS

Animal Treatment

Male Sprague-Dawley (SD) rats (Charles River Lab, Wilmington, MA), weighing 170–190 g, were maintained on a standard rat diet and tap water ad libitum. After the rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (65 mg/kg body weight), diabetes was induced by a single injection, *via* the tail vein, of streptozotocin (STZ, Sigma, St. Louis, MO) (45 mg/kg body weight) dissolved in 0.05 mol/L citrate buffer (pH 4.5). Blood glucose levels were elevated (426±39 mg/dl) two days after STZ injection, but were maintained between 160-200 mg/dl for the duration of the study by insulin administration. Insulin was essential to assure that ketosis and weight loss were insignificant. Glucose monitoring was performed using an automated analyzer (Lifescan Inc., Milpitas, CA). Beginning the day after either STZ injection or sodium citrate buffer injection (in control rats) Resveratrol (30 mg/kg daily), CORM-3 (30 mg/kg daily), CoPP (3 mg/kg once a week), or CoPP (3 mg/kg once a week) plus SnMP (20 mg/kg, three times a week) were administered i.p. for 8 weeks. CORM-3 was dissolved in water and administered daily at a dose of 2 mg/100 gm body wt., inactive CORM-3 (iCORM-3) was used as a control.

There were six groups of rats: A) controls, B) STZ-saline, C) STZ- Resveratrol, D) STZ-CORM-3, E) STZ-CoPP, and F) STZ-CoPP-SnMP with a minimum of 4 rats/group. Food intake did not change in the rats treated with Resveratrol, CORM-3 or CoPP. The Animal Care and Use Committee of New York Medical College approved all experiments. Animals were sacrificed and the thoracic aorta was removed and divided in two sections; one for immunohistochemical study was fixed in 4% paraformaldehyde and the other was immediately frozen in liquid nitrogen for eventual determination of HO-1 and HO-2 protein expression, heme content and HO activity.

The selection of CORM-3 stems from our evaluation of several CO-donors synthesized in the laboratory of Dr. Falck at the University of Texas. Preliminary studies were performed to evaluate the efficacy of the following CO donors with regard to CO release: CORM-3, tricarbonyl chloro (glycinato) ruthenium (ii), CORM-4, tricarbonyl chloro (glutamic acidato) ruthenium (ii), CORM-5, tricarbonyl chloro (lysinato) ruthenium (ii) and CORM-6, tricarbonyl chloro (phenyl alanato) ruthenium (ii). For *in vitro* analysis, each CORM was dissolved in 10 mM K^+ phosphate buffer (pH 7.4) at various concentrations, sealed in gas-tight amber vials, and an internal standard made of isotopically-labeled CO ($^{13}C^{18}O$, Aldrich-Sigma) was injected. After 1 and 24 hours, the amount of CO released into the headspace of each vial was determined *via* gas chromatography/mass spectroscopy analysis as previously described [42]. At the highest concentration (1 mM) examined, CORM-3 released the greatest amount of CO [25].

Tissue Preparation for Ultrastructural Analysis

Aorta segments were removed and immediately fixed in 2% glutaraldehyde in phosphate buffer (pH 7.4). Sections were then cut and observed by a Philips CM10 transmission electron microscopy as previously described [25]. Immunohistochemical analysis was performed as previously described [25].

Detection and Quantification of Circulating Endothelial Cells (CEC) and Endothelial Cells Fragmentations

For the immunomagnetic isolation and quantification of CEC, we used monodispersed magnetizable particles (Dynabeads CELLection Pan Mouse IgG kit) obtained from Dynal (Lake Success, NY). Typically, 100 µl of bead suspension was noncovalently coated with 10 µg/ml of RECA-1 (Novus Biologicals, Littleton, CO), a pan-rat endothelial cell specific monoclonal antibody, and CEC were determined [25]. Under microscopic examination, there was evidence of cellular fragmentation and debris, which was bound by the RECA-1 coated beads and fluoresced after staining with acridine, but was too small to be considered as whole cells. Quantification of these fragments yielded an average of 5.4±1.3 fragments/ml in controls rats.

Cell Culture Conditions and Western Blot Analysis of peNOS, pAKT, ICAM-1, VCAM-1

Human dermal microvessel endothelial cells were grown in a MCDB131 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% FBS, 10 ng/mL epithelial growth factor, and 1 µg/mL hydrocortisone (Sigma-Aldrich, Saint Louis, MO). The cells were incubated at 37ºC in a 5% CO_2 humidified atmosphere and maintained at subconfluency by passage with Trypsin-EDTA (GIBCO-BRL). Cells were harvested using a cell lysis buffer as previously described

[9]. The lysate was used to measure the protein levels of HO-1 and HO-2, peNOS (Ser 1177), pAKT (Ser 473), CD54 (ICAM-1) and CD106 (VCAM-1) as previously described [37].

Determination of HO Activity

Frozen aorta were pulverized under liquid nitrogen and placed in homogenization buffer (10 mM phosphate buffer, 250 nM sucrose, 1 mM EDTA, 0.1M pMSF and 0.1% tergitol pH 7.5). Homogenates were centrifuged at 27,000xg for 10 min. at 4°C. Heme oxygenase activity was determined as previously described [28]. Bilirubin formation was calculated using an extinction coefficient of 40 mM^{-1} cm^{-1} between 464 nm and 530 nm.

Caspase Activity Assay and Adiponectin Measurements

Caspase-3 activity was determined by colorimetric assay (ApoTarget Kit, BioSource, Camarillo, CA), following the manufacturer's protocol as previously described [41]. The high molecular weight (HMW) form of Adiponectin was determined using an ELISA assay (Pierce Biotechnology, Inc., Woburn, MA). This kit selectively measures the biologically active HMW form of adiponectin.

Statistical Analyses

The data are presented as mean ± standard error (SE) for the number of experiments. Statistical significance between experimental groups and between different study conditions was determined by using a two-way ANOVA followed by the Fisher's exact test; $p<0.05$ was considered significant.

RESULTS

Effect of Resveratrol, CORM-3 and CoPP on aorta HO activity, serum adiponectin levels and circulating endothelial cell fragmentation.

We examined the effect of long-term treatment, eight weeks, with Resveratrol, CORM-3 and CoPP on HO activity in the aorta of diabetic rats. As seen in Fig. (**1A**), Resveratrol, CORM-3 and CoPP administration increased the levels of HO activity in STZ-treated diabetic animals compared to controls.

However, the increase in HO activity as a result of the administration of either Resveratrol or CORM-3, while significant ($p<0.01$), was less than that after CoPP treatment ($p<0.001$). HO activity decreased ($p<0.05$) in the aorta of saline treated STZ diabetic animals when compared to control animals (Fig. **1A**). The increase in HO activity that resulted from the administration of inducers of HO activity was blocked by the co-administration of SnMP (results not shown).

There was significant ($p<0.05$) decrease in adiponectin levels between STZ-treated and control rats. Resveratrol increased adiponectin from 3.1±0.26ug/ml to 7.75±0.21ug/ml in diabetic rats ($p<0.05$). Similarly, both CoPP and CORM-3 administration caused a significant increase in serum adiponectin levels, Fig. (**1B**), ($p<0.05$). The increase in adiponectin seen with CORM-3, was similar to that seen with the other HO-1 inducers. As seen in Fig. (**1C**), in a

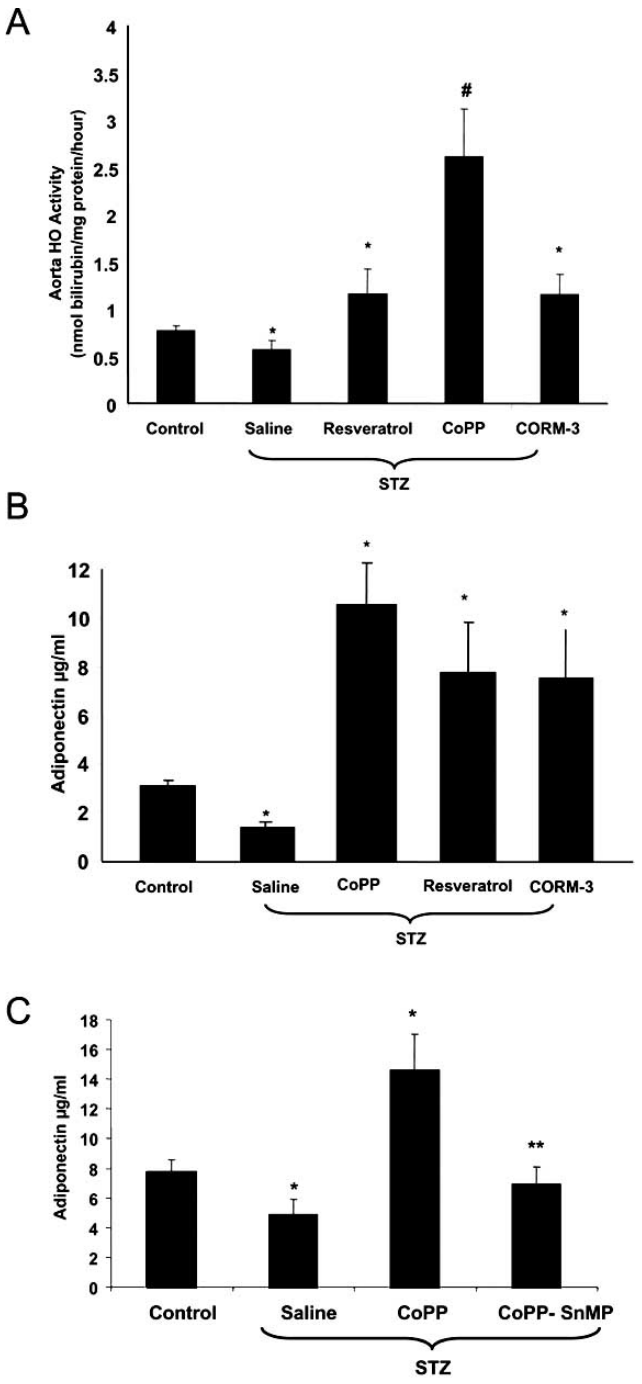

Fig. (1). A) Effect of HO-1 inducers, Resveratrol, CoPP and CORM-3 (see methods for dosing regimen) on HO activity in diabetes rats (n=4) *$p<0.05$ control *vs.* saline treated diabetic rats, **$p<0.01$ *vs.* diabetes-CORM-3 and Resveratrol, ***$p<0.001$ vs. diabetes-CoPP. Fig. (**1B**) and (**C**): Adiponectin levels in plasma of control, diabetic rats and diabetic rats treated with B) Resveratrol, CORM-3 and CoPP *$p<0.05$ control *vs.* diabetes treated rats and C) Effect of inhibition of HO activity by SnMP on HO-1 induction of adiponectin. (n=6). *$p<0.05$ control *vs.* saline treated hyperglycemic animals, **$p<0.01$ control *vs.* diabetes-CoPP and ***$p<0.05$ CoPP-SnMP *vs.* CoPP-diabetes.

separate experiment, using the most potent inducer of HO-1, CoPP, administration of CoPP increased serum adiponectin from 7.8±0.76ug/ml to 14.61±2.4ug/ml ($p<0.01$). The decrease in serum adiponectin levels between control and saline treated hyperglycemic rats seen in Fig. (**1B)** was confirmed Fig. (**1C**). To further ascertain the direct effect of HO-1 expression on the increases in serum adiponectin, we examined the effect of SnMP, on serum adiponectin levels. Rats were treated with CoPP in combination with SnMP as described in the method section, the serum level of adiponectin was decreased ($p<0.01$) to control levels when compared with CoPP-treated animals as a result of inhibition of HO activity, Fig. (**1C**). HO-1-mediated increases in adiponectin were associated with a decrease in the inflammatory cytokines TNF-α and IL-6. TNFα decreased from 161±58 pg/ml to 108±27 pg/ml, $p<0.05$, IL-6 declined from 261±74 pg/ml to 120±43 pg/ml,$p<0.05$, STZ-treated and CoPP-treated respectively (Results not shown).

Effect of CoPP on CEC Levels

Using immunomagnetic isolation, we identified a well-defined population of cells, which reproducibly stained positive for VWF, CD34 and UEA-1, in peripheral blood of normal and diabetic rats [25]. Fig. (**2**) shows that very few endothelial cells (mean 7± 3 cells/ml) were detected in vehicle-treated control rats while a large number of CEC (58 ± 4 cells/ml; $p<0.003$ compared to control) were seen in blood obtained from diabetic rats. CoPP administration was associated with a significant decrease in the number of circulating endothelial cells, $p<0.002$ compared to saline treated diabetic rats. Under light microscope examination, every endothelial cell fragment attached to the immunomagnetic beads yielded a fluorescent band in the corresponding location. The basal levels of endothelial cell fragmentation in controls were compared to those seen in diabetic rats. There was a ~9-fold increase in cell fragmentation in diabetic rats compared to controls (data not shown). This beneficial effect of CoPP on CEC levels was blocked by the inhibition of HO activity and of adiponectin release into circulation by the administration of SnMP (Fig. **2**).

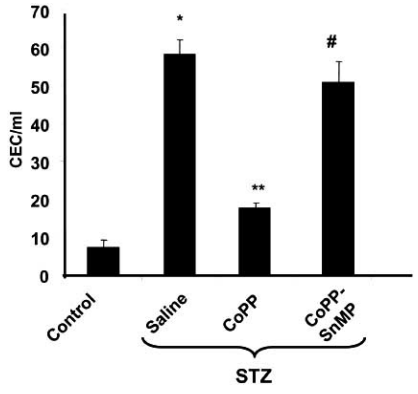

Fig. (2). The number of CECs in control and diabetic rats,(n=6), (*$p< 0.003$ control *vs.* untreated diabetic rats,** $p<0.002$ CoPP *vs.* untreated diabetic rats, #$p<0.05$ SnMP-CoPP *vs.* CoPP treated diabetic rats.

Effect of CoPP on TM and $CD31^+$ Expression

Since adiponectin levels were increased following CoPP administration, we examined the effect of CoPP on the restoration of vascular TM and $CD31^+$ levels. Immunohistochemical staining for TM was carried out in aorta isolated from untreated and CoPP-treated diabetic animals. Staining appeared brown and was localized within the endothelial cell cytoplasm. TM staining was strong in the intima of control rats (Fig. **3A,** a) while diabetic rats demonstrated a moderate to weak staining (Fig. **3A**, b). CoPP treatment restored TM expression in diabetic rats to the level of staining seen in aorta from control animals (Fig. **3A**, c). Optical density analysis of immunohistochemical staining provided quantification of the changes in TM expression (Fig. **3B**). The levels of TM were decreased ($p<0.05$) in diabetic animals when compared to control animals. TM levels were restored to levels in control animals after CoPP treatment (Fig. **3B**). In control animals, strong $CD31^+$ immunoreactivity was seen in the aorta (Fig. **3C**, a). In diabetic animals, $CD31^+$ staining was either weak or absent (Fig. **3C**, b); however, treatment with CoPP restored the pattern to that seen in controls (Fig. **3C**, c). $CD31^+$ levels were decreased ($p<0.05$) in diabetic animals and restored by CoPP treatment to levels found in control animals (Fig. **3D**).

Effect of CO on Adiponectin and CEC and EC Fragmentation and pAKT, AKT, peNOS and BcL-2

Since the diabetic conditions decrease adiponectin and increases endothelial cell sloughing and cell death, we further compared the effect of CO on the levels of adiponectin on both of these parameters. As seen in Fig. (**4A**), plasma serum levels of adiponectin were decreased in STZ induced diabetes and that daily administration of CORM-3 increases adiponectin levels compared to untreated diabetic rats ($P<0.05$). Administration of the inactive iCORM-3 in releasing CO, did not increase adiponectin (Results not shown). The increase in adiponectin in CORM-3 treated diabetic rats is associated with a reversal in CEC. As seen in Fig. (**4B**), administration of CORM-3 decreases CEC compared to untreated diabetes. The number of CEC in diabetic rats were 58.3±5.4 cell/ml compared to 23.3±2.9 cell/ml in CORM-3 treated diabetic rats.

Effect of CO on Endothelial Cell Fragmentation

Under microscopic examination, there was evidence of cellular fragmentation and debris, which was bound by the RECA-1 coated beads and fluoresced after staining with acridine, but was too small to be considered as whole cells [25]. Quantification of these fragments (Fig. **4C**) yielded an average of 5.8±1.3 fragments/ml in controls and 48.3±6.2 fragments/ml in untreated diabetic rats ($p=0.005$). Administration of CORM-3 to diabetic rats prevented endothelial fragmentation by 50.3% in diabetic rats; only 24±3.3 fragments/ml was seen in CORM-3-treated diabetic rats ($p<0.02$ vs. untreated diabetics). The above results suggest that the increase in CO is associated with prevention of EC death and restoration of $CD31^+$ and TM. Therefore, we examined the effect of CORM-3 on the levels of signaling protein in the aorta of diabetic rats. As seen in Fig. **4D**, increased levels of CO resulted in increased protein levels of pAKT and peNOS

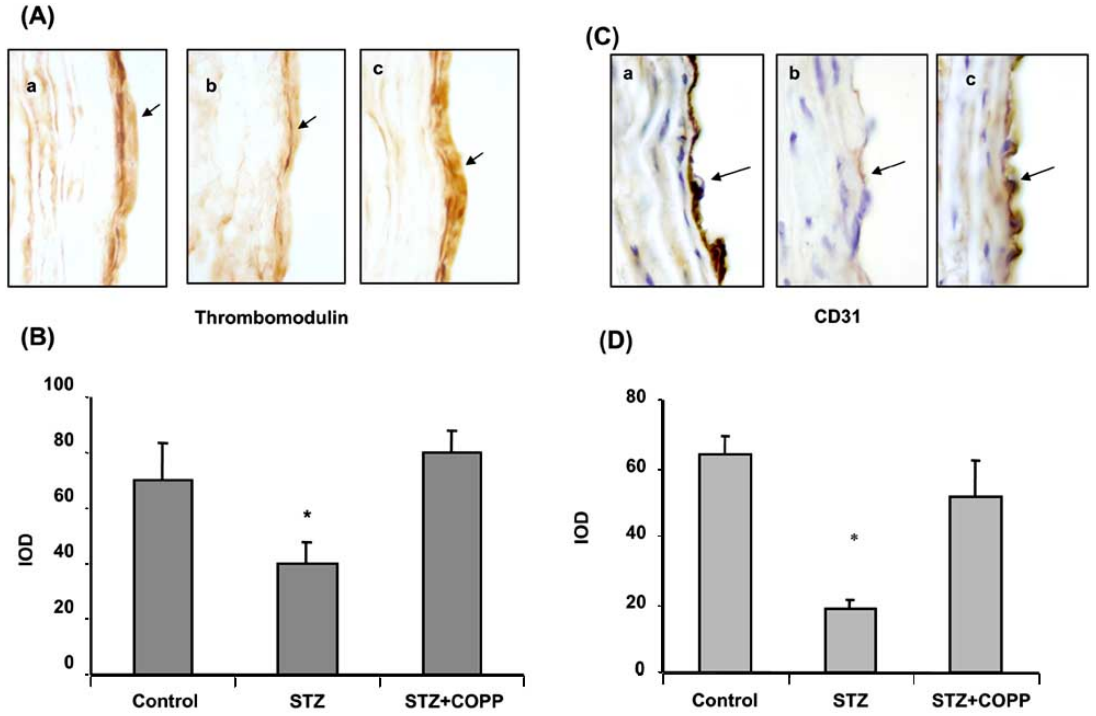

Fig. (3). A) Immunohistochemical staining of TM EC (arrows) from control (a), diabetic (b) and diabetes-CoPP (c) B). Optical density analysis of TM staining in diabetes and its restoration by CoPP, n=4, *p<0.05 control vs diabetic animals C) Immunohistochemical staining of $CD31^+$ EC (arrows) from control (a), diabetic (b) and CoPP-diabetes (c) and Optical density analysis D) demonstrates the loss of $CD31^+$ staining in diabetes and preventing its loss by CoPP; *p<0.05 *vs.* STZ treated; (n=4).

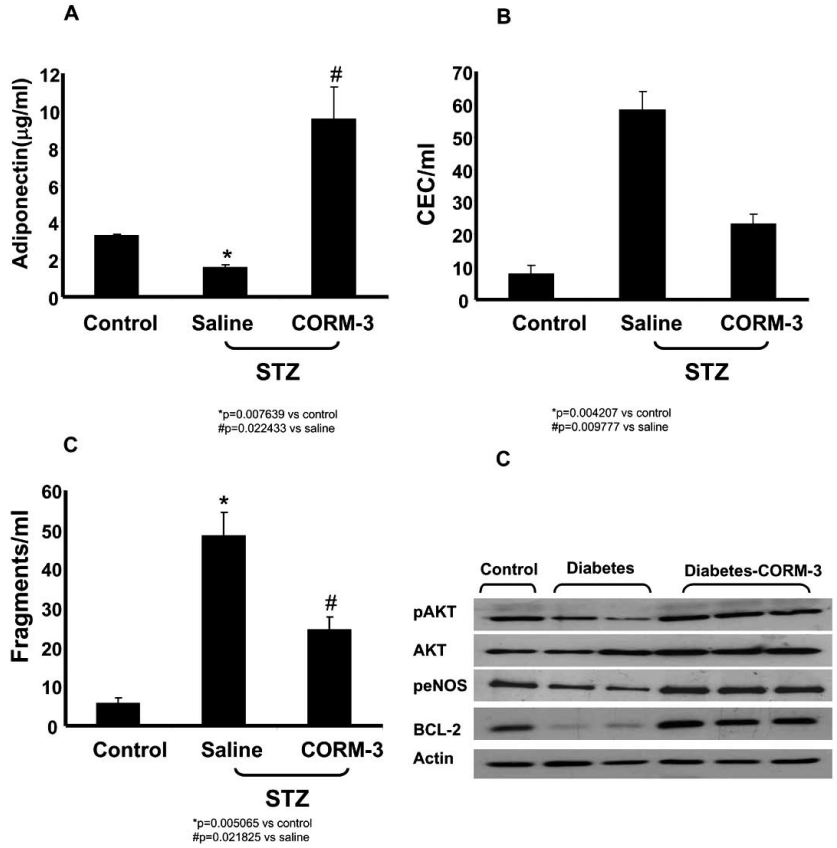

Fig. (4). A) Adiponectin levels in plasma of control, diabetic rats and diabetic rats treated with CORM-3, **B)** CECs in control and diabetic rats, (n=6); **C)** Endothelial cell membrane fragments in blood obtained form control, diabetic and CORM-3- diabetes, n=5 rats, *p<0.005 vs. control; #p< 0.02 vs. diabetes vs. control, and **D)** Effect of CO on peNOS, BCL-2, AKT, pAKT and α-actin in control and diabetic rats treated with CORM-3, representative Western blot analysis is displayed (n=5).

suggesting that prevention of EC fragmentation was associated with the restoration of kinase activity. In addition levels of the anti-apoptotic protein BcL-2 were increased (Fig. **4D**).

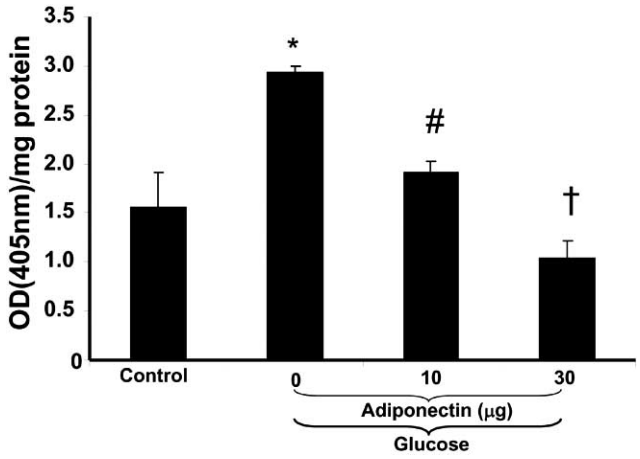

Fig. (5). Caspase-3 activity in endothelial cells exposed to glucose 25mM for 48 hours in the presence or absence of adiponectin. (n=4), (*p<0.05 compared to control, #p<0.05 compared to 25mM glucose + p<0.001 compared to 25mM glucose).

Effect of Adiponectin on Hyperglycemia-Mediated Increase in Caspase 3 Activity *In Vitro*

We have previously shown that high glucose levels cause microvessel endothelial cell death [9, 41]. We, therefore, investigated the effect of perturbations in adiponectin levels on caspase 3 activity using cells that were deprived by culture in 1% FBS prior to treatment with 25 mM D-glucose. As seen in (Fig. **5**) endothelial cells exposed to 25 mM glucose displayed a significant increase in caspase 3 activity compared to controls (p<0.05). In endothelial cells exposed to glucose in the presence of adiponectin, caspase 3 activity was decreased in a concentration-dependent manner, suggesting that adiponectin prevented a glucose-mediated increase in caspase 3 activity (p<0.001). The maximum adiponectin-mediated inhibition of caspase activity was achieved (p<0.001) at the highest concentration examined, 30μg/ml (Fig. **5**).

Effect of Adiponectin on TNF-α -Mediated Expression of ICAM-1 and VCAM-1

To further clarify the role of adiponectin in vascular protection mechanisms and endothelial cell survival, we evaluated, *in vitro*, the effect of adiponectin on TNF-mediated expression of adhesion molecules and endothelial cell death. Endothelial cells, serum deprived by culturing in 1% FBS, were treated with TNF-α (100 ng/ml) for 24 hours in the presence and absence of adiponectin. As seen in (Fig. **6A** and **B**), cells treated with TNF-α in the absence of adiponectin displayed a significant increase in ICAM-1 and VCAM-1 protein levels (p<0.01 and 0.05 respectively). In contrast, cells treated with TNF-α for 24 hours in the presence of adiponectin (10 and 30 μg/ml) showed a significant decrease in ICAM-1 and VCAM-1 protein levels compared to TNF-α treatment alone (Fig. **6B** and **6C**). As seen from densitometry analysis (Fig. **6B**, **C**), TNF-α increased the expression of both ICAM-1 and VCAM-1 compared to control endothelial cells (p<0.01 and 0.05 respectively). In contrast, cells treated with TNF-α and with adiponectin resulted in an adiponectin dose-dependent inhibition of adhesion molecule protein expression. These findings demonstrate *in vitro* that increased levels of adiponectin protect against the TNF-α-induced expression of inflammatory proteins.

Effect of Adiponectin on Activation of AKT and peNOS

To determine whether the protective effect of adiponectin is related to signaling proteins in the suppression of caspase 3 and adhesion molecule expression. We measured *in vitro* the effect of adiponectin (10 and 30 μg/ml) on activation of AKT. Following adiponectin treatment, a concentration-dependent increase in pAKT was observed (Fig. **7A**). Pre-incubation of endothelial cells with the p13 kinase/AKT pathway inhibitor LY294002 blocked the adiponectin-mediated increase in pAKT at Ser 473 (Fig. **7A**). As seen in the densitometry analysis (Fig. **7B**), adiponectin, in a dose-dependent manner and with a maximum effect at 30 μg/ml, increased pAKT proteins levels compared to control endothelial cells (p<0.001). These findings indicate that increased levels of adiponectin are associated with increased levels of pAKT. A similar pattern was seen for the phosphorylation of eNOS at Ser 1179. This effect was partially blocked by LY294002, (Fig. **7B**) Thus, the ability of adiponectin to augment vascular repair *in vivo* may be due, in part, to an increase in the levels of pAKT and peNOS.

DISCUSSION

This study documents a novel role of adiponectin (HMW) as well as the existence of a temporal HO-1-adiponectin relationship that appears to play a central role in providing cellular vascular cytoprotection. Four observations substantiate this conclusion. First, the HO-1 inducer, Resveratrol , in similar manner to CoPP, caused robust increases in adiponectin levels in a STZ animal model of Type 1 diabetes (Fig. **1B**). This observation along with the finding that CO can act independently in providing vascular cytoprotection and increase serum levels of adiponectin focuses on the role of HO-1 on vascular protection. In addition, CORM-3 administration inhibited endothelial cell fragmentation, suggesting that CORM-3 causes vascular protection *via* a mechanism that involved an increase in serum levels of adiponectin, thus, suggesting that CO may have antioxidant properties through an increase in levels of adiponectin. We have previously reported that CORM-3 in the presence of SnMP decreased endothelial cell sloughing in diabetic rats [28]. These data together with the results we describe here imply that the heme degradation product, CO, is directly involved in this process. Recently, Guo *et al.* reported that total adiponectin levels are markedly decreased in the STZ animal model of diabetes [43]. Our results are in agreement with this report in that, adiponectin levels decreased in diabetic animals when compared to controls (Fig. **1B** and **1C**). In addition, Fukushima *et al.* have reported decreased adiponectin levels in STZ induced diabetic mice that could be alleviated by the hydrodynamic injection of the adiponectin gene [44]. Adiponectin is also decreased in Type 2 diabetes and coronary artery disease [3] and in obese diabetic mice [45-47].

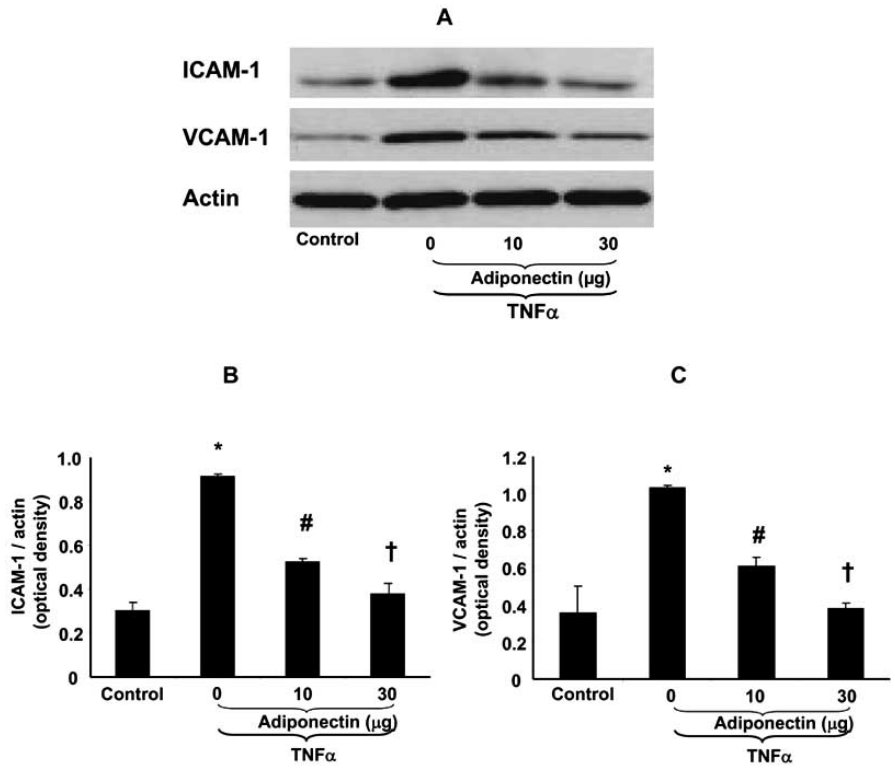

Fig. (6). A) Western blot and densitometry analysis of ICAM-1 and VCAM-1 in EC exposed to TNF-α (100ng/ml) for 24h in presence or absence of Adiponectin. **B)** and **C)** The expression of α-actin is an index of the adequacy of sample loading and Quantitative densitometric evaluation of ICAM-1, and VCAM-1, normalized relative to α-actin. Results are expressed as mean ± SE., n=3. 5B: $p<0.01$ control *vs*. TNF-α, #$p<0.01$ TNF-α *vs.* adiponectin, +$p<0.005$ TNF-α *vs.* adiponectin 5C *$p<0.05$ control *vs*. TNF-α, #$p<0.01$ TNF-α *vs*. adiponectin, + $p<0.005$ TNF-α *vs*. adiponectin.

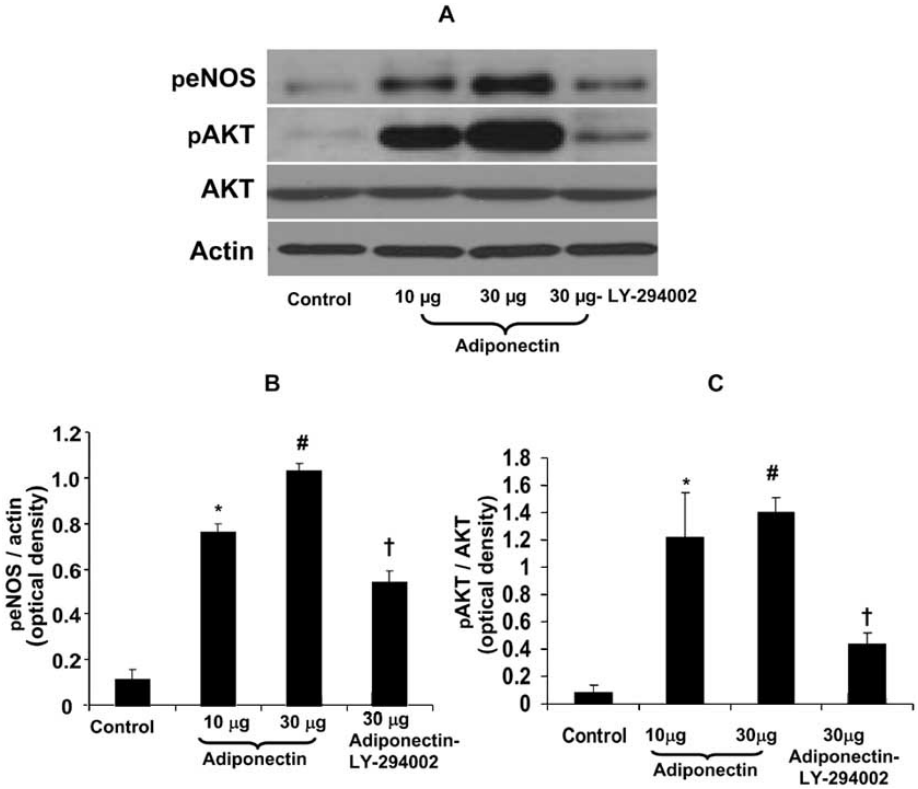

Fig. (7). A) Western blot and densitometry analysis of pAKT and peNOS proteins in endothelial cells exposed to adiponectin for 60 minutes. Data are representative of 6 separate experiments. **B)** and **C)**: Mean band density normalized of peNOS relative to actin and pAKT, AKT respectively (n=6). (*, #, $p< 0.001$, compared to untreated).

Second, the increase in adiponectin levels that resulted from either CO or CoPP, administration was associated with a decrease in endothelial cell sloughing, which was paralleled by a decrease in endothelial cell death and fragmentation (Figs. **3-5**). This effect was blocked by SnMP delineating the presence of a temporal relationship between HO-1 expression, HO activity and adiponectin. This temporal relationship appears central to the beneficial changes in vascular resistance to diabetes-mediated vascular injury that we report. This hypothesis is bolstered by the observation that increased levels of HO-1 expression, HO activity and adiponectin were associated with a concomitant increase in the levels of $CD31^+$ and TM in the vascular wall. Third, in a series of *in vitro* experiments increasing adiponectin levels inhibited both the glucose-mediated increase in caspase 3 activity and the TNF-α mediated increase in expression of the adhesion molecules, ICAM-1 and VCAM-1. Fourth, adiponectin caused a significant dose-dependent reduction in the TNF-α mediated induction of ICAM-1 and VCAM-1 *in vitro*. We have previously reported that the TNF-α mediated increased levels of adhesion molecules can be prevented by the overexpression of HO-1 [38, 48]. The results described above clearly demonstrate for the first time that the changes in serum adiponectin that parallel increased HO activity and HO-1 expression define a central role of a temporal HO-1/ adiponectin relationship in vascular cytoprotection. It is also noteworthy that these effects were achieved with physiological doses of adiponectin (Figs **1B** and **C**).

Reduced endothelial cell expression of $CD31^+$ and TM, important indicators of endothelial cell death, has been associated with the progression of atherosclerotic heart disease [32, 33, 49] while restoration of their expression represents a reversal of this process by the prevention of atherosclerosis and myocardial infarction [34, 50]. Our results are in accord with previous studies showing that the downregulation of TM and $CD31^+$ expression in endothelial cells occurs in coronary atherosclerosis in humans [32] and in diabetic rats [25]. The diminished vascular endothelial cell function that occurs in experimental [25, 41, 51] and human diabetes [32, 35] is accompanied by decreased levels of CD31 and TM, increased endothelial cell death and sloughing [9, 41] and a reduction in EPC number and function [52], which further impacts the integrity of the intact endothelial cell lining. Prevention of the diabetes-induced decrease in endothelial cell expression of TM provides an insight into the mechanism(s) of the anti-atherosclerotic and cardio-protective properties of adiponectin [3, 5, 53]. The adiponectin-mediated increase in TM expression in the aorta of diabetic rats suggests that adiponectin is involved in vascular repair of the endothelium. We hypothesize that the parallel increases in HO-1 expression and adiponectin levels are crucial in providing the endothelial cell with resistance to ROS generated from hyperglycemia mediated cell death and/or increases in endothelial progenitor cells for vascular repair. Regardless of the mechanism, however, increases in the levels of the CO and adiponectin appear crucial to vascular repair and function as evidenced by increased TM, peNOS and pAKT levels, all of which are clinically relevant in diabetes. The increase in TM limits neointimal formation and endothelial cell dysfunction [32]. It should be noted, however, that while the induction of peNOS and pAKT *in vitro* protects endothelial cell function, the effect of CO is also confirmed *in vivo*.

The existence of a close correlation in changes in HO-1 expression, CO and adiponectin levels may explain the direct effect of adiponectin on the observed increase in peNOS and pAKT. Serine/threonine protein kinase AKT/PKB is recognized as a key regulator of cell endothelial cell growth and migration [54]. Although, adiponectin was reported to increase endothelial cell death [55], others have shown that adiponectin is protective *via* activation of pAKT through the promotion of the survival/proliferation of endothelial cells [56]. We show that adiponectin activates pAKT and eNOS in a dose dependent manner (Figs. **6A** and **B**). Our results support those of Ouchi *et al.* and others who, in a series of elegant studies, have reported that adiponectin is critical for endothelial cell survival and function [57-59] *via* the activation of eNOS and AKT, and the inhibition of cell adhesion.

Various isoforms of adiponectin have been reported of which the HMW form appears to have a significant role to play in cellular metabolism [60]. In agreement with these results the HMW from of adiponectin was increased concomitant with the elevation of HO-1 expression. In addition, adiponectin blocked the TNF-α mediated increase in the levels of ICAM-1 and VCAM-1 in a dose dependent manner (Figs. **5B**, **5C**). Previous reports [38, 48] have indicated that increased expression of HO-1 can block TNF-α mediated increases in adhesion molecules. Thus it appears that the parallel increases in HO-1 expression, HO activity and adiponectin can be extended to a cellular response to pro-inflammatory cytokines and that the changes reported here in HO-1 expression and adiponectin may play a central role in cellular cytoprotection, as reflected by decreases in the levels of TNF-α and IL-6. It should be noted that the levels of adiponectin used in these *in vitro* studies were similar to those found *in vivo* after administration of CoPP (Fig. **1B, C**), thus further strengthening the link between increased levels of HO-1 protein expression and adiponectin.

We have previously shown that upregulation of HO-1 in diabetic rats provided cardio- and vascular protection against ROS [14]. This was attributed to an increase in HO-1-derived CO and bilirubin production *via* a mechanism that involved an increase in adiponectin, EC-SOD and eNOS [25, 26, 28]. In addition, HO-1 upregulation also increases the expression of eNOS and superoxide dismutase [26], which contribute to an increase in antioxidants and a reduction in oxidized protein levels in serum [26]. The increase in adiponectin as a result of CORM-3 adminis-tration in Type 1 diabetes suggests, however, that CO is directly involved in cellular protection, similar to that described for type 2 diabetes [46, 61] and in an animal model of streptozotocin (STZ)-induced diabetes [28]. This is further strengthened by the fact that SnMP blocks the CoPP mediated increase in adiponectin, thus implicating changes in HO activity in this process. The seminal finding of the parallel perturbations in HO-1 and adiponectin described in this report suggests the direct involvement of CO and provides a new and unique explanation of the mechanism(s) involved in the increased HO-1 expression and HO activity protection against the detrimental effects of ROS in experimental diabetes.

The increase in adiponectin expression brought about by inducers of HO-1 or CO releasing molecules, CORM-3, resulted in vascular protection as reflected by the reduction in endothelial cell death and fragmentation, the restoration of TM and CD31 levels pAKT, peNOS and BcL-2. These results extended our previous studies in the heart [14] and are of considerable interest from both a clinical and a basic science perspective, clearly defining the existence of HO-1-adiponectin axis, and the involvement of CO, that can be manipulated to provide vascular cytoprotection in critical areas of cell damage associated with cardiovascular disease and diabetes.

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ABBREVIATIONS

HO-1 = Heme oxygenase-1

CO = Carbon monoxide

CORM-3 = Tricarbonylchloro glycinato ruthenium II

CoPP = Cobalt protoporphyrin

STZ = Streptozotocin

CEC = Circulating endothelial cells

TM = Thrombomodulin

peNOS = Phospho eNOS

pAKT = Phospho-AKT

ROS = Reactive oxygen species

ICAM-1 = Intercellular adhesion molecule

VCAM-1 = Vascular cell adhesion molecule

EC-SOD = Extracellular superoxide dismutase

O_2^- = Superoxide

SnMP = Tin mesoporphyrin

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