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TMPDP, a tetramethylpyrazine derivative, protects vascular endothelial cells from oxidation damage by hydrogen peroxide

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A novel ligustrazine derivative, tetramethylpyrazine diphenylmethyl piperazidine (TMPDP), prepared by hybridization and bioisosteric replacement of the molecular structure of TMP, was studied for its protective effects on oxidative damage of human umbilical vein endothelial cells (HUVECs) in response to hydrogen peroxide (H_2O_2). The antioxidative effect of TMPDP was assessed by the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) test. Cell viability was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The activity of lactate dehydrogenase (LDH), superoxide dismutase (SOD) and glutathione peroxidase (GSH) and the content of malondialdehyde (MDA) in cells were determined by commercial kits. The intracellular formation of reactive oxygen species (ROS) and the concentration of free intracellular calcium ($[Ca^{2+}]_i$) were determined using DCFH-DA assay and with fura-2/AM fluorimetry, respectively. Results showed that TMPDP had a moderate antioxidative effect against DPPH. Cell viability was decreased markedly by exposure to H_2O_2. Introduction of TMPDP, however, significantly increased cell viability, markedly reduced LDH release from cells and decreased lipid peroxidation in response to H_2O_2 treatment. These effects of TMPDP were accompanied by increased activity of the endogenous antioxidant enzymes, SOD and GSH, reduced production of ROS and reduced intracellular concentration of Ca^{2+}. These results suggest that TMPDP protects HUVECs against oxidative damage by scavenging ROS and regulates intracellular calcium concentration. This might have important implications for the development of new agents for the effective treatment of vascular disease.

1. Introduction

There is increasing evidence that oxidation stress of endothelial cells, an imbalance in the cellular production and elimination of reactive oxygen species (ROS), is involved in the pathophysiology of several vascular diseases such as atherosclerosis, diabetes and hypertension (Heo et al. 2007). In particular, H_2O_2-induced oxidative stress leads to the death of endothelial cells as well as many other types of cell. Oxidative stress can damage the structure of DNA, induce peroxidation of membrane lipids and proteins, and damage the fluidity and permeability of the cell membrane (Yamagishi et al. 2008). This reduces the ability of cells to regulate the concentration of intracellular ions, especially calcium.

Tetramethylpyrazine (TMP), is a major active component of the traditional Chinese medicinal herb *Chuanxiong* (*Ligusticum wallichii* Franchat) and is widely used in China like calcium channel antagonist for the treatment of ischemic diseases, both cerebrovascular and cardiovascular (Hintz and Ren, 2003; Xu et al. 2003; Kao et al. 2006). However, pharmacokinetic studies have shown that TMP displays low bioavailability and is metabolized quickly *in vivo* with short half-life, $T_{1/2} = 2.89$ h (Ye et al. 1996). TMP also shows accumulating toxicity when it is administered frequently. Structure-activity relationship studies indicate that the pyrazine ring of TMP largely determines its pharmacodynamics, while the substituted groups (four methyl groups) primarily appear to govern its pharmacokinetics and toxicity (Liu et al. 2003). Therefore, it is possible that modification of the methyl position of TMP by drug-like groups and pharmacophores of some sort could improve the pharmacokinetics of TMP.

Calcium channel antagonists such as cinnarizine, flunarizine, and lomerizine are very important cardiovascular drugs. The piperazine ring acts as a link in the molecular structures of these drugs and is considered to be an active functional group in the drugs. By hybridization and bioisosteric replacement, we generated a series of novel ligustrazine derivatives, combining a piperazine with some pharmacophores or drug-like groups, such as substituted benzyl, cinnamyl, bisphenylmethyl or ligustrazinyl to form new integrated structures. Our early studies showed that, in comparison with TMP, ligustrazine derivatives exhibit lower EC_{50} values for protecting endothelial cells from damage caused by hydrogen peroxide (Cheng et al. 2007). Some ligustrazine derivatives also presented anti-platelet aggregation activity, especially tetramethylpyrazine diphenylmethyl piperazidine (TMPDP), which was synthesized by replacing the methyl group of the TMP molecule with the (4,4′-fluorine)diphenyl-methyl-piperazidine-methylium group of flunarizine (Fig. 1).

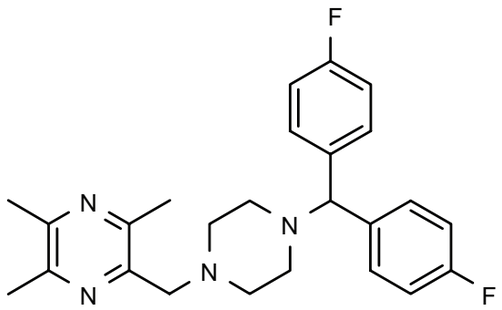

Fig. 1: Chemical structure of TMPDP

In this study, we investigated the effects of TMPDP on damage to human umbilical vein endothelial cells (HUVECs) in response to H_2O_2.

2. Investigations and results

2.1. In vitro antioxidative potential of TMPDP

The antioxidative potential of TMPDP was assessed against DPPH. Results showed that TMPDP had a moderate antioxidative effect against DPPH, stronger than equal molar concentrations of TMP at 120 μmol/ml and similar to TMP at 15, 30, and 60 μmol/ml (Fig. 2).

2.2. TMPDP increases viability of HUVECs in response to H_2O_2

The effects of TMPDP on the growth of oxidative injury to HUVECs in response to H_2O_2 were investigated by the MTT method. The exposure of HUVECs to H_2O_2 at 150 μmol/L for 6 h or 12 h resulted in significant reductions in cell viability (Fig. 3). Pretreatment of the cells with TMPDP for 12 h, however, attenuated the effect of H_2O_2 on cell viability in a dose-dependent manner.

2.3. TMPDP inhibits LDH release from HUVECs damaged by H_2O_2

Treatment of HUVECs with 150 μmol/L H_2O_2 for 6 h or 12 h caused a significant increase of LDH release (an indicator of membrane integrity) (Fig. 4). Pre-treatment of the cells with various concentration of TMPDP for 30 min prior to incubation with H_2O_2 significantly inhibited LDH release induced by H_2O_2 in a TMPDP concentration-dependent manner.

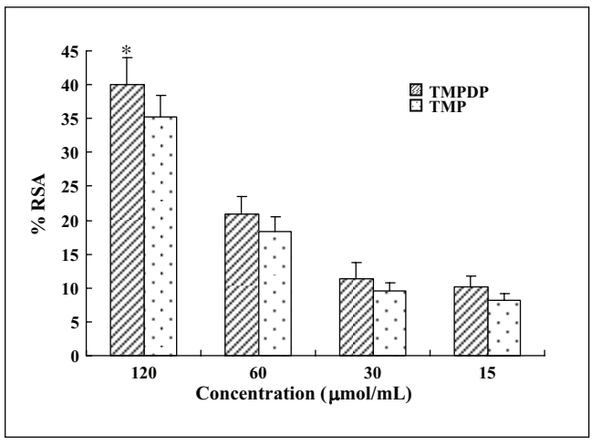

Fig. 2: Effect of TMPDP against DPPH test. For each concentration of TMPDP and TMP tested (15, 30, 60 and 120 μmol/ml), reduction of DPPH radical was followed by monitoring decrease of absorbance at 516 nm. Data of % RSA expressed as mean ± S.D. (n = 6). $^{*}P < 0.05$, compared with 120 μmol/ml TMP-treated group

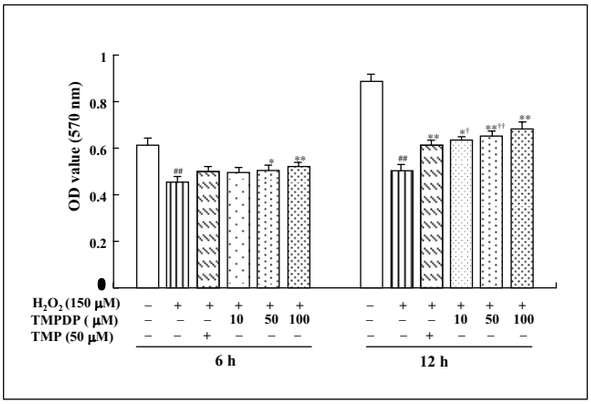

Fig. 3: Effect of TMPDP on HUVECs viability in response to H_2O_2. Cells incubated with TMPDP for 30 min and then exposed to H_2O_2 (150 μmol/L) for 6 h or 12 h before cell viability was determined by MTT method. All data expressed as mean ± S.D. (n = 8). $^{\#\#}P < 0.01$, compared with unstimulated cells; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with H_2O_2-stimulated cells; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, compared with cells treated with H_2O_2 +TMP

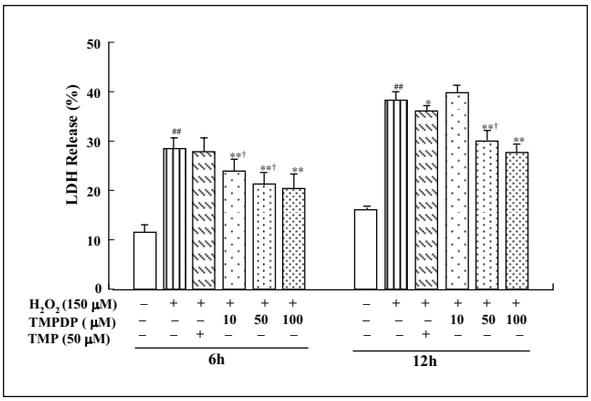

Fig. 4: Effect of TMPDP on H_2O_2-induced release of LDH in HUVECs. Cells incubated with TMPDP or TMP for 30 min and then exposed to 150 μmol/L H_2O_2 for 6 h or 12 h before LDH release was measured. All data expressed as mean ± S.D. (n = 8). $^{\#\#}P < 0.01$, compared with unstimulated cells; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with H_2O_2-stimulated cells; $^{\dagger}P < 0.05$, compared with cells treated with H_2O_2 +TMP

2.4. Effect of TMPDP on lipid peroxidation and free radical scavenging

Incubation of HUVECs with 150 μmol/L H_2O_2 for 12 h caused a significant increase of MDA content and a marked decrease of SOD and GSH-Px activities (Table). TMPDP pre-treatment significantly attenuated the increase in MDA content and decreased SOD and GSH-Px activity in response to H_2O_2 in a TMPDP concentration-dependent manner. In comparison to the H_2O_2-only group, in the TMPDP treated groups the amounts of MDA were reduced by 16.3 % (10 μmol/L), 22.3 % (50 μmol/L) and 26.9 % (100 μmol/L), respectively. The activities of SOD were increased by 11.6 % (50 μmol/L) and 19.8 % (100 μmol/L) respectively. The activities of GSH-Px were increased by 14.6 % (10 μmol/L), 43.2 % (50 μmol/L) and 59.2 % (100 μmol/L) by TMPDP.

Table: Effects of TMPDP on MDA level, SOD activity and GSH-Px activity in HUVECs injured by H_2O_2 ($\bar{x} \pm s$, $n = 8$)

Groups	Dose (μmol/L)	MDA (nmol/ml)	SOD (U/ml)	GSH-Px (U/ml)
Normal	—	2.26 ± 0.50	23.02 ± 1.55	134.2 ± 6.3
H_2O_2	150	$3.68 \pm 0.72^{**}$	$16.07 \pm 1.12^{**}$	$64.4 \pm 9.4^{**}$
TMP + H_2O_2	50 + 150	$3.00 \pm 0.79^{\#}$	$17.13 \pm 0.73^{\#}$	$76.4 \pm 9.7^{\#\#}$
	10 + 150	$3.08 \pm 0.52^{\#}$	16.85 ± 0.63	$73.8 \pm 10.3^{\#}$
TMPDP + H_2O_2	50 + 150	$2.86 \pm 0.75^{\#}$	$17.94 \pm 1.34^{\#\#}$	$92.2 \pm 8.5^{\#\#\dagger\dagger}$
	100 + 150	$2.69 \pm 0.45^{\#\#}$	$19.25 \pm 0.81^{\#\#}$	$102.5 \pm 10.3^{\#\#}$

$^{**}P < 0.01$, Significant difference compared with normal control group
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, Significant difference compared with H_2O_2 group
$^{\dagger\dagger}P < 0.01$, Significant difference compared with TMP group

2.5. Effect of TMPDP on cellular production of ROS

The production of ROS in HUVECs in response to H_2O_2 was examined using the fluorescence probe DCF. We found that H_2O_2 treatment resulted in an increase in DCF fluorescence intensity compared with cells treated with cultured medium only (Fig. 5). The increase in the DCF signal was suppressed by pretreatment of the cells with 10, 50 or 100 μmol/L TMPDP. These results indicate that TMPDP effectively inhibited the production of cellular total ROS.

2.6. Effect of TMPDP on intracellular Ca^{2+} concentration

The fluorescent Ca^{2+} indicator, fura 2-AM, was used to measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). In the presence of Ca^{2+} in the culture medium, addition of TMPDP for 10, 15, 20 and 30 min, respectively reduced the intracellular concentration of Ca^{2+} in response to subsequent H_2O_2 stimulation (Fig. 6). The presence of TMP showed a similar effect on $[Ca^{2+}]_i$ as TMPDP, though to a lesser extent, especially with treatment for 30 min.

3. Discussion

The vascular endothelium forms a cellular interface between the blood and underlying tissue, encompassing the entire vasculature and the heart. Vascular endothelial cells participate in numerous regulatory tasks, such as control of vascular permeability and blood coagulation, regulation of vascular smooth muscle tone and blood pressure, and influencing the growth, inflammatory processes and angiogenesis of the cell (Brutsaert 2003; He 2005). Endothelial dysfunction has been implicated in the initiation and propagation of vascular disease processes including atherosclerosis, hypertension, cardiac hypertrophy and congestive heart failure (Gonzalez and Selwyn 2003). Oxidative stress induced by ROS, e.g., superoxide, hydrogen peroxide (H_2O_2), peroxynitrite, etc., is believed to be a key reason for endothelial cell dysfunction (Lum and Roebuck 2001). In the present study, we have demonstrated that pretreatment of the cells with TMPDP effectively protects HUVECs from H_2O_2-induced damage in a concentration-dependent manner. Overproduction of ROS could break down mitochondrial function through the mitochondrial ATP-sensitive potassium channels and the mitochondrial permeability transition pore (mPTP) (Hausenloy and Yellon 2003). The mPTP is an early event in cell apoptosis, while maintenance of mitochondrial membrane potential (MMP) is a critical primary determinant of blood vessel endothelial cell survival (Schönfeld and Wojtczak 2008). In this study, TMPDP showed a moderate antioxidative effect against DPPH, and a greater beneficial effect than TMP at equal concentrations at 120 μmol/ml. In HUVEC cells, TMPDP significantly decreased intracellular ROS generation, suggesting that the protective effect of TMPDP is probably

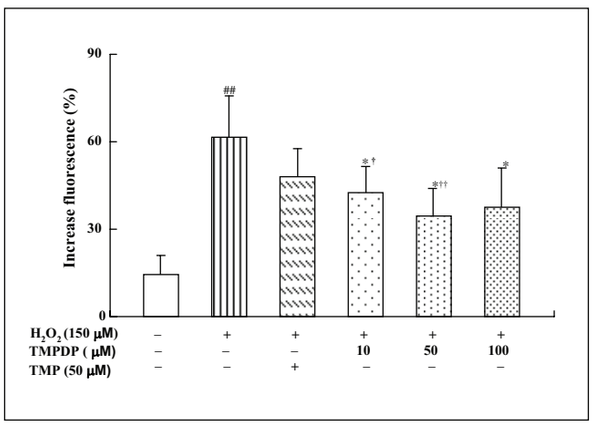

Fig. 5: Effect of TMPDP on ROS production in HUVECs in response to H_2O_2. Cells labeled with non-toxic fluorescence dye, DCFH-DA, and treated with different concentrations of TMPDP or TMP (50 μmol/L) for 30 min followed by exposed to H_2O_2 (150 μmol/L) for 12 h. Fluorescence intensities of DCF due to oxidation of DCFH by cellular ROS (generated by H_2O_2) detected (Ex = 485 nm and Em = 535 nm). ROS produced quantified by DCF fluorescence and depicted as percentage of fluorescence increase. All data expressed as mean ± S.D. (n = 8). H_2O_2 (150 μmol/L) increased oxidized DCF levels, which were significantly decreased by the presence of TMPDP. $^{\#\#}P < 0.01$, compared with the unstimulated cells; $^{*}P < 0.05$, compared with H_2O_2-stimulated cells; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, compared with cells treated with H_2O_2 +TMP

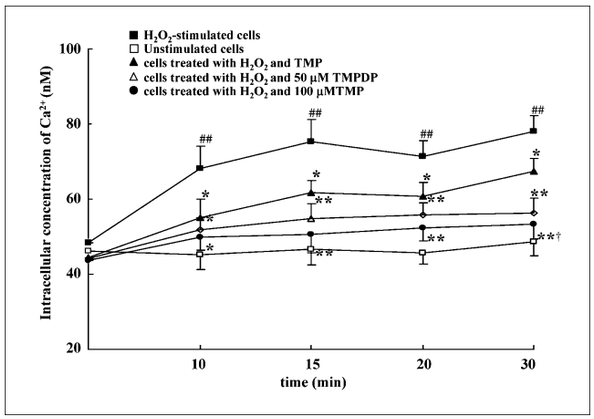

Fig. 6: Effect of TMPDP on intracellular Ca^{2+} concentration in HUVECs injured by H_2O_2. Fura 2-AM loaded cells treated with TMPDP at 10, 50, 100 μmol/L, or 50 μmol/L TMP for 10, 15, 20, 30 min respectively and followed by incubation with H_2O_2 for another 30 min. Unstimulated cells group treated with culture medium only. Fluorescence measurements converted to $[Ca^{2+}]_i$ by determining maximum and minimum fluorescence. All results expressed as means ± S.D. (n = 8). $^{\#\#}P < 0.01$, compared with the unstimulated cells; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with H_2O_2-stimulated cells; $^{\dagger}P < 0.05$, compared with cells treated with H_2O_2 +TMP

associated with scavenging the formation of intracellular ROS. Antioxidant enzymes, such as SOD and GSH-Px, have been shown previously to be involved in enhancing the antioxidant defenses in endothelial cells (Muzykantov 2001). SOD scavenges superoxide radicals by converting them to hydrogen peroxide which is then converted to water by catalase and GSH-Px. The level of MDA reflects the extent of cell damage by oxidative stress. Our results show that TMPDP reduces the decline in activity of SOD and GSH-Px in HUVECs in their response to H_2O_2, suggesting that the inhibitory effect of TMPDP on the formation of cellular ROS is likely to be related to the restoration of endogenous antioxidation. Moreover, it has been previously suggested that ROS affect Ca^{2+} signaling in various cell types, including endothelial cells, cardiomyocytes, cardiac fibroblasts, pulmonary arterial myocytes and neural cells (Waypa et al. 2002; Fujii et al. 2005; Kang et al. 2006; Kazantsev 2007; Florea and Blatter 2008). Exogenous addition of hydrogen peroxide can evoke an increase in cytosolic Ca^{2+} in CNS pericytes (Kamouchi et al. 2007). Our results here demonstrate that the inhibition of ROS by TMPDP greatly modulates intracellular Ca^{2+} concentration in HUVECs.

The homeostasis of Ca^{2+} in cytoplasm is maintained by extracellular Ca^{2+} entry and Ca^{2+} release from the intracellular calcium stores. Oxidant stress may promote the entry of Ca^{2+} into vascular endothelial cells and inhibit store-operated Ca^{2+} influx, thus causing a steady increase of intracellular $[Ca^{2+}]_i$ (Florea and Blatter 2008). An excessive increase in the intracellular concentration of Ca^{2+} would result in Ca^{2+} overload and thus cause damage to HUVECs by inducing cytoskeleton breakdown and DNA fragmentation. Many early studies have shown that the action of TMP on the cardiovascular system may be attributed to its involvement in modulating intracellular Ca^{2+} level by inhibiting Ca^{2+} entry and/or Ca^{2+} release (Zhu et al. 2006). In this study, cells treated with TMPDP for 10, 15, 20 and 30 min, respectively, all showed inhibition of intracellular accumulation of Ca^{2+} induced by H_2O_2. Furthermore, TMPDP shows a greater effect than TMP on Ca^{2+} antagonism, improving the viability of injured HUVECs, and on inhibiting LDH release and scavenging ROS.

In conclusion, the results showed that TMPDP prevented HUVECs from oxidative injury induced by H_2O_2 by improving the activities of endogenous antioxidases, scavenging ROS and regulating intracellular calcium concentration. Hence, TMPDP may have the potential to be developed further as an effective agent for the treatment of cardiovascular disease.

4. Experimental

4.1. Materials

MTT (3,(4,5-dimethylthiazole-2-yl) 2,5-diphenyl-tetrazolium bromide), fura-2 acetoxymethylester, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 6-carboxy-2′, 7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma (St. Louis, Mo, USA). Endothelial Cell Medium (ECM) was purchased from ScienCell Research Labs (San Diego, CA, USA). LDH, SOD, GSH-PX and MDA detection kits were purchased from Nanjing Jiancheng Bioengineering Institute. All other chemicals used were of analytical grade and were from Shanghai Sangon Biological Engineering Technology & Sciences Co.Ltd. (Shanghai, China).

4.2. Estimation of radical scavenging activity (RSA) by the DPPH test

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) was used as stable radical. The antioxidative potential of TMPDP was studied against DPPH (Spasova et al. 2008). For each concentration of TMPDP and TMP tested (15, 30, 60 and 120 μmol/L), the reduction of DPPH radical was followed by monitoring the decrease of absorbance at 516 nm. The absorption was monitored at the start and at 10 min using an Agilent 8453 UV-Visible spectrophotometer (Agilent Technologies, USA). The results are expressed as % RSA = [Abs516 nm (t = 0) -Abs516 nm (t = 10 min) × 100/Abs516 nm (t = 0)].

4.3. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from Sciencell Research Laboratories (Sciencell, USA). The cells were cultured in plastic flasks coated with polylysine and grown in ECM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FBS in a humidified atmosphere of 5% CO_2 at 37 °C.

Stock solutions of TMPDP or TMP were dissolved in DMSO and diluted with ECM supplement (0.1% (V/V) DMSO), and were used at the concentrations indicated. HUVECs were incubated with TMPDP at final concentrations of 10, 50, 100 μmol/L respectively for 30 min before treatment of the cells with 150 μmol/L H_2O_2 for 6 or 12 h. Cells were also treated with TMP 50 μmol/L as a positive control in the same as with TMPDP.

4.4. MTT assay for cell viability

Cell viability was measured by an MTT assay. Briefly, after 6 or 12 h exposure to H_2O_2, MTT dye (20 μl) was added to each well at a final concentration of 0.5 mg/ml. After 4 h incubation, 200 μl of solubilization/stop solution, DMSO was added for dissolving the formazan crystals and the absorbance was read using a Spectra Rainbow (Tecan, Austria) microtiter plate reader at a wavelength of 570 nm.

4.5. Detection of cellular lactate dehydrogenase (LDH) release

HUVECs in 96-well plates were pretreated with TMPDP or TMP for 30 min, and then stimulated with H_2O_2 (150 μmol/L) for 6 h or 12 h in the presence or absence of TMPDP. LDH release into the supernatant of the cells was measured using a commercial available kit according to the manufacturer's protocol. To determine the intracellular LDH activity, cells were washed with PBS twice, and lysed in 500 μl lysis buffer (150 mM NaC1, 150 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100), and the supernatants were obtained by centrifugation at 10,000 rpm at 4 °C for 10 min. LDH release (%) = (LDH activity in supernatants) / (LDH activity in supernatants + LDH activity in total cells) × 100% (Hu et al. 2007).

4.6. Malondialdehyde (MDA) assay and enzyme activity

HUVECs were pretreated with TMPDP or TMP for 30 min, and then stimulated with H_2O_2 (150 μmol/L) for 12 h in 96-well plates in the presence or absence of TMPDP. Cells were lysed with extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). Cell lysates of each well were collected and used for determination of enzyme activity. The levels of total lipid peroxidation products, MDA, were assayed by the thiobarbituric acid method, based on quantifying malondialdehyde-reactive products at 532 nm (Wang et al. 2007). Superoxide dismutase (SOD) activity was measured using a commercial kit based on a hydroxylamine assay developed from a xanthine oxidase assay. Nitrite was produced on oxidation of hydroxylamine by cellular superoxide anion free radical ($O_2^{-}\cdot$). The removal of $O_2^{-}\cdot$ by SOD is accompanied by a decrease in absorbance at 550 nm, which in turn quantifies SOD activity. Glutathione peroxidase (GSH-Px) activity was measured with a commercial kit based on GSH-Px-mediated consumption of reduced glutathione (GSH). GSH was determined by reaction with dithiodinitrobenzoic acid to yield a chromophore with a maximum absorbance at 412 nm.

4.7. Measurement of cellular reactive oxygen species (ROS)

Intracellular formation of ROS was assessed as described previously using the oxidation sensitive dye DCFH-DA as the substrate (Qian et al. 2008). Briefly, HUVECs growing in fluorescence microtiter 96-well plates were loaded with 20 μmol/L DCFH-DA in Hanks balanced salt solution (HBSS) and incubated in the dark for 30 min. The cells were then treated with TMPDP at 10 μmol/L, 50 μmol/L, 100 μmol/L, or 50 μmol/L TMP and incubated for another 30 min. After three washes of the cells with PBS, 150 μmol/L H_2O_2 was added. The formation of 2′,7′-dichlorofluorescin (DCF) due to oxidation of DCFH in the presence of various ROS was analyzed 30 min before and after H_2O_2 introduction with a 1420 Vitor3 Multilabel Counter (Perkin Elmer Life Science, USA) at an excitation wavelength (Ex) of 485 nm and an emission wavelength (Em) of 538 nm. The percentage variation of ROS in each well was calculated by the formula $[(Ft_{30}\text{-}Ft_0)/Ft_0 \times 100]$. Ft_0 is the fluorescence intensity before H_2O_2 and Ft_{30} is the fluorescence intensity after 30 min of H_2O_2 addition.

4.8. Measurement of intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$)

Intracellular Ca^{2+} levels were measured by the method described by Kamouchi et al. (2007). Confluent HUVECs were dispersed with 0.25 % trypsin and 0.02 % EDTA, and washed three times with HBSS containing 0.2 % BSA. Cells were incubated with fura-2 acetoxymethylester (at a final concentration of 5 μmol/L) at 37 °C for 45 min in HBSS. After washing, the cells were incubated for 20 min in a physiological salt solution containing 0.5 % BSA and 10 mM glucose. The cells were collected by centrifugation at 1000 rpm for 10 min and washed twice with D-Hank's solution with 0.2 % BSA to remove the Fura 2-AM in solution. The Fura 2-AM loaded cells were treated with TMPDP at 50 μmol/L, 100 μmol/L, or 50 μmol/L TMP for 10, 15, 20, 30 min, respectively, before incubation with H_2O_2 for another 30 min. Fluorescence intensities of fura-2-loaded suspended cells were measured at 37 °C using continuous rapid alternating excitation from dual monochromators (340 and 380 nm) with emission at 510 nm with a 1420 Vitor^3 Multilabel Counter. The ratio of the fluorescence intensities at 340 and 380 nm (F = F340/F380) was calculated. Fluorescence measurements were converted to calcium concentration by determining the maximal fluorescence (F_{max}) with Triton X-100 (final concentration 0.1%), followed by the minimal fluorescence (F_{min}) with 15 mM EGTA, at pH 10.5 for 15 min. The formula, $[Ca^{2+}]_i = Kd\ [(F\text{-}Fmin)] / (Fmax\text{-}F)]$, was used for conversion (assuming that Kd for the Fura-2-Ca^{2+} complex was 224 nm at 37 °C).

4.9. Statistical analysis

The results were expressed as means ± S.D. of data from at least three independent experiments and were analyzed by one-way analysis of variance (ANOVA), the Newman-Keuls $P < 0.05$ being considered as statistically significant.

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