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Sildenafil induces angiogenic response in human coronary arteriolar endothelial cells through the expression of thioredoxin, hemeoxygenase and vascular endothelial growth factor

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Abstract

This study was undertaken to investigate the effect of phosphodiesterase-5 (PDE5) inhibitor, sildenafil, on angiogenic response in human coronary arteriolar endothelial cells (HCAEC). The cells exposed to sildenafil (1–20 μM) demonstrated significantly accelerated tubular morphogenesis with the induction of thioredoxin-1 (Trx-1), hemeoxygenase-1 (HO-1) and VEGF. Sildenafil induced VEGF and angiopoietin specific receptors such as KDR, Tie-1 and Tie-2. This angiogenic response was repressed by tinprotoporphyrin IX (SnPP), an inhibitor of HO-1 enzyme activity. Sildenafil below 1 μM has no angiogenic effect as evidenced by reduced tuborogenesis. Sildenafil along with SnPP inhibited both VEGF and Angiopoietin-1 (Ang-1) protein expression. Therefore our results demonstrated for the first time that sildenafil is a very potent pro-angiogenic factor.

Keywords: Angiogenesis; Hemeoxygenase; Thioredoxin; VEGF; Angiopoietin-1; Tuborogenesis

1. Introduction

The well-studied sex drug, sildenafil citrate (Viagra) help men having heart-pounding sex, as well as it helps to decrease the stress on the hearts by selectively inhibiting cyclic 3′,5′-monophosphate (cGMP)-specific phosphodiesterase 5 (PDE5). This drug is widely used for the treatment of erectile dysfunction in men (Cheitlin et al., 1999; Kloner and Jarow, 1999). Its pharmacological action is due to prolonging the signaling actions of nitric oxide (NO) in penile smooth muscle (Andersson, 2001). Interestingly, Ockaili et al. reported a pronounced infarct size-reducing effect of sildenafil in an in vivo rabbit model of coronary occlusion (Ockaili et al., 2002). There are other reports also (including ours) which documented reduced infarct size by sildenafil in mice and rat hearts subjected to global ischemic/reperfusion (I/R) injury (Salloum et al., 2003; Das et al., 2002). Sildenafil is therefore suggested to have preconditioning-like cardioprotective effect. Studies suggest that sildenafil exerts cardioprotection through nitric oxide (NO) generation that potentially activate GC (Guanylate Cyclase) resulting in enhanced formation of cGMP. cGMP eventually activates PKG, which subsequently opens mitochondrial kATP channels resulting in delayed cardioprotection (Kukreja et al., 2005). Again a potential target for NO treatment of stroke and myocardial infarction is angiogenesis (Ziche and Morbidelli, 2000; Fukuda et al., 2004). Treatment with sildenafil was found to induce capillary-like tube formation in a culture of brain-derived endothelial cells. In addition this report (Zhang et al., 2003) also demonstrated sildenafil mediated increased levels of VEGF in the ischemic brain. Thus, there appears to be an interrelationship between sildenafil mediated NO, VEGF and angiogenesis in the stroke model. Angiogenesis is significantly controlled by two families of growth factors, the VEGF and angiopoietin families, as well as endothelial cell interaction with extracellular matrix

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(Yancopoulos et al., 2000). The most notable being vascular endothelial growth factor (VEGF), which has been chiefly associated with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells. Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) are the endothelial specific tyrosine kinase receptors of VEGF through which its effects are primarily mediated (DeVries et al., 1992; Millauer et al., 1994). Two other angiogenic factors, the angiopoietins 1 and 2 (Ang-1 and Ang-2), have been found to regulate the maturation of new blood vessels from the proliferated endothelial cells (Witzenbichler et al., 1998; Ray et al., 2000). Tie-1 and Tie-2 comprise another family of endothelial specific receptor tyrosine kinases, Ang-1 and Ang-2 being the specific ligands for Tie-2.

In the present study here we report the effects of sildenafil on the protein expression profiles of VEGF and the Angiopoietin–Tie system using human coronary arteriolar endothelial cells (HCAEC) as an in vitro angiogenesis model. We also examined the redox protein, thioredoxin-1 (TRX-1), and hemeoxygenase-1 (HO-1) expression with sildenafil treatment. The relative expression of the various components of these two systems as well as apparent relationships between TRX-1 and HO-1 seems to suggest a probable sequence of involvement during in vitro angiogenesis by sildenafil treatment, as proposed in our model. Such relationships may potentially be utilized in formulating strategies for sequential gene therapy to achieve clinically relevant brain and/or myocardial angiogenesis.

2. Materials and methods

2.1. Endothelial cell culture

Human coronary arteriolar endothelial cells (HCAEC) were obtained from Cambrex (Walkersville, MD) and they were serially passaged. Cells were maintained in a culture medium, EGM-2 supplemented with growth factors and antibiotics according to company specifications (Cambrex, Walkersville, MD, USA).

2.2. Sildenafil treatment

Confluent HCAEC were plated on plastic 100 mm dishes supplemented with cell media, with specifications as mentioned above and subjected to different concentration of sildenafil treatment (0.1 nM, 100 nM, 1 μM, 10 μM and 20 μM for tube formation) for 24 h in a 5% CO_2 incubator. After determination of the dose we used 10 and 20 μM of sildenafil to determine the protein expression profile. For some experiments cells were treated with SnPP (5 μM), inhibitor of HO-1 activity to determine whether sildenafil mediated expression of Trx-1 followed by HO-1 activity has any influence on VEGF expression.

2.3. In vitro tube formation on Matrigel

100 μl of ice cold Matrigel (BD Bioscience, Bedford, MA) was coated on a 12 well cell culture plate (from Costar) as a base for tube formation. After allowing the gel to settle for 30 min in a 37 °C, 5% CO_2 incubator, the endothelial cells (5×10^4) were seeded onto the Matrigel and incubated overnight at 37 °C, CO_2 incubator. The wells were treated with different concentrations of sildenafil as mentioned earlier immediately after the cells were seeded. After 18 h exposure to sildenafil the extent of tube formation was recorded by the phase-contrast microscope (magnification × 200) with a digital camera.

2.4. Western blot analysis for Trx-1, HO-1, VEGF, Ang-1, KDR, Flt-1, Tie-1 and Tie-2

For Western blot analysis, protein is isolated from the cells by removing the medium and washing the cultured plate with PBS and scrapping the cells with RIPA buffer (Boston BioProducts, Worcester, MA). After scrapping the cells with RIPA buffer the lysate was passed through 21G needle syringe to shear the DNA and the lysate was precipitated with PMSF. Following precipitation the lysate was spinned at 10,000 ×*g* for 10 min at 4 °C. The supernatant obtained was the total lysate. Total protein concentrations were determined using BCA (bicinchoninic acid) protein assay kit (Pierce, Rockville, IL). Cell lysates (40 μg) were run on polyacrylamide electrophoretic gels (SDS-PAGE) typically using 10–14% (acrylamide to bis ratios) based on the protein of interest. Separated proteins were electrophoretically transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) using a semidry transfer system (Bio-Rad, Hercules, CA). Protein standards (Bio-Rad, Hercules, CA) were run in each gel. The blots were blocked in Tris-buffered saline/Tween-20 (TBS-T containing 20 mM Tris base, pH 7.6, 137 mM NaCl, 0.1% Tween-20) supplemented with 5% (wt/vol) non-fat dry milk for 1 h. Blots were incubated overnight at 4 °C with the specific primary antibodies. Membranes were washed three times in TBS-T before incubation for 1 h with horse radish peroxidase-conjugated secondary antibody diluted 1:2000 in TBS-T and 5% (wt/vol) non-fat dry milk. Following incubation membranes were washed three times with TBS-T for 10 min each, blots were treated with Enhanced Chemi-Luminescence (ECL from Amersham, Life Science, Inc, Arlington Heights, IL) reagent and the required proteins were detected by autoradiography for variable lengths of time with Kodak X-Omat film. All the samples were tested for non-specific labeling. Negative and positive controls were run to validate the results.

3. Results

3.1. Tubular morphogenesis of human coronary arteriolar endothelial cells (HCAEC) after sildenafil exposure

Human coronary endothelial cells plated on the surface of Matrigel after sildenafil treatment form tube-like structures. The formation of capillary network of tubular structure was extremely prominent when the cells were exposed to 20 μM sildenafil (Fig. 1). The cells started forming tubular structure with 1 μM sildenafil however complete structures were more prominent when 20 μM sildenafil was used. Sildenafil with lower dosage such as 0.1 and 100 nM showed no effect on tuborogenesis. In separate experiments the cells were preincubated with SNPP (5 μM) to demonstrate sildenafil mediated

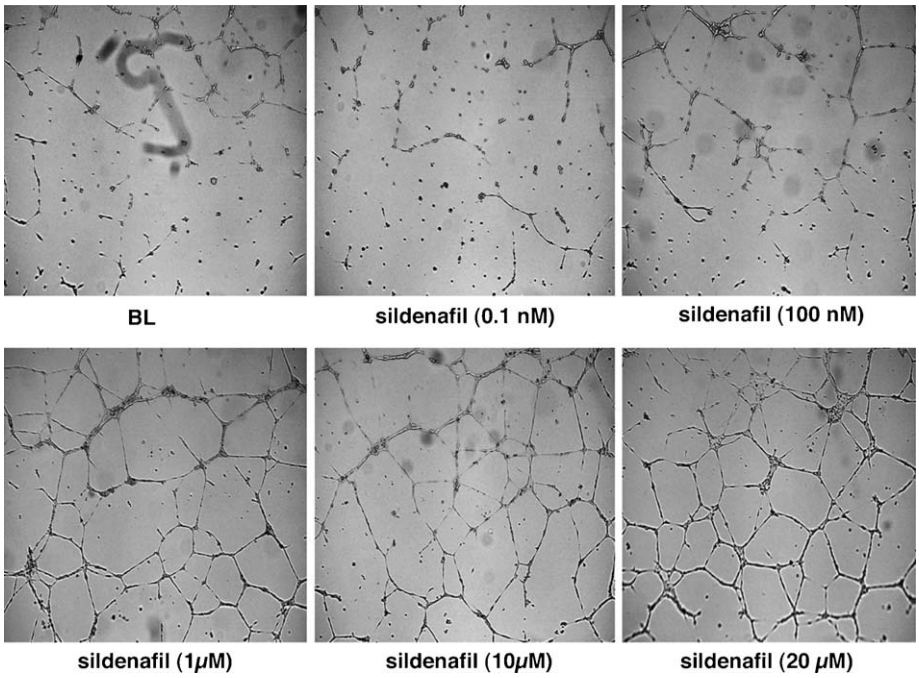

Fig. 1. Sildenafil treatment induced tube-like structure. Human coronary arteriolar endothelial cells (HCAEC) were plated onto Matrigel once they were confluent. Cells were exposed to various concentration of Sildenafil ranging from 0, BL (Baseline), 0.1 nM, 100 nM, 1 μM, 10 μM, 20 μM. The tube formation is very prominent when cells are exposed to 10–20 μM.

tuborogenesis involves HO-1 activity. As expected cells pretreated with SnPP displayed inhibition of sildenafil mediated tubular morphogenesis of HCAEC (not shown).

3.2. Analysis of VEGF system

3.2.1. Western blot analysis

Western blot analysis revealed a prominent VEGF, KDR bands which were significantly induced dose dependently in HCAEC when exposed to sildenafil treatment. VEGF protein expression was found to be increased by 1 and 2 fold on treatment with 10 and 20 μM sildenafil respectively as compared to the non-treated control group. Similarly KDR protein expression, the most active receptor of VEGF was also found to be increased by 2.5 and 4.5 folds with 20 μM sildenafil (Fig. 2). There was no effect on the other VEGF receptor, Flt-1 by sildenafil treatment in HCAEC. Again the sildenafil mediated VEGF expression was found to be inhibited with SnPP significantly as shown in Fig. 5. Thus this result documents that sildenafil mediated VEGF expression may depend on HO-1 activity.

3.3. Analysis of Ang–Tie system

3.3.1. Western blot analysis

Protein analysis documented significant increase in Ang-1 and its receptors Tie-1 and Tie-2 (Fig. 3) after sildenafil treatment. The protein expression of Ang-1, Tie-1 and Tie-2 was found to be dose dependent. Ang-1 was found to be increased by 1 and 1.5 folds compared to the non-treated cells with 10 and 20 μM sildenafil respectively. Cells treated with 10 and 20 μM of sildenafil demonstrated 2 and 4 folds increase in Tie-1 protein expression whereas Tie-2 demonstrated 1.8 and 5 folds increase with 10 and 20 μM of sildenafil respectively.

3.3.2. Effect of hemeoxygenase (HO-1) inhibitor, SnPP on Trx-1, Ang-1 and VEGF

Sildenafil (20 μM) was found to induce Trx-1 (1.9 fold) (Fig. 5), HO-1 (3 fold) (Fig. 4) and VEGF (2 fold) (Fig. 2)

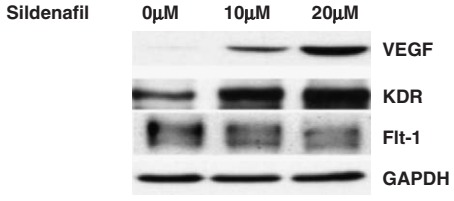

Fig. 2. Representative Western blot showing the effect of sildenafil (10 and 20 μM) on the expression of VEGF, KDR and Flt-1 (VEGF system) and β-actin in HCAEC. VEGF was expressed as 40 kDa band whereas KDR and Flt-1 proteins were expressed as 150 and 200 kDa respectively. GAPDH was used as loading control.

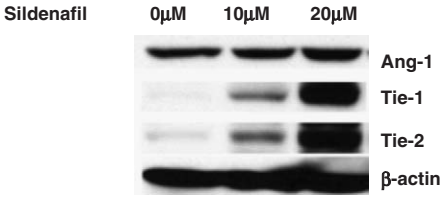

Fig. 3. Representative Western blot showing the effect of sildenafil (10 and 20 μM) on the expression of Ang-1, Tie-1 and Tie-2 (Ang–Tie system) and β-actin in HCAEC. Ang-1 was expressed as 60 kDa band whereas Tie-1 and Tie-2 proteins were expressed as 140 kDa respectively. β-actin was used as loading control.

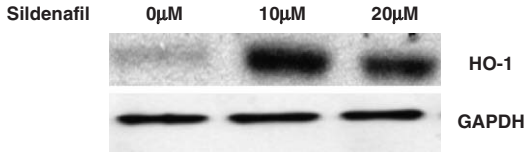

Fig. 4. Representative Western blot showing the effect of sildenafil (10 and 20 μM) on the expression of HO-1 also known as HSP-32 and GAPDH in HCAEC. HO-1 was expressed as 32 kDa band. GAPDH was used as loading control.

significantly compared to non-treated cells. Our previous report has shown the mechanism that HCAEC exposure to Trx-1 increased the expression of HO-1 and VEGF in the Trx-1 treated cells (HCAEC) compared to the control cells (Kaga et al., 2005). SnPP (5 μM) demonstrated strong inhibition of sildenafil induced Ang-1 and VEGF protein expression (Fig. 5) but no change in Trx expression as it seems to be upstream regulator of HO-1.

4. Discussion

In the present study, we report sildenafil mediated induction of cytosolic thioredoxin-1 (Trx-1) along with hemeoxygenase-1 (HO-1) in human coronary arteriolar endothelial cells. Recent studies suggest the contribution of this Trx system in the upregulation of HO-1 protein levels as well as HO-1 promoter activity under conditions associated with inflammation and increased oxidative stress (Kaga et al., 2005; Wiesel et al., 2000). Transfection of cells with human Trx-1 is found to increase the overall production of VEGF in MCF-7 breast cancer, HT-29 colon cancer, and WEHI7.2 lymphoma cells (Ejima et al., 2002). We have recently documented that transgenic mouse heart overexpressing Trx-1 is resistant to ischemia/reperfusion injury as evidenced by improved post-ischemic ventricular function and reduced myocardial infarct size when compared to the corresponding wild type control myocardium (Gallegos et al., 1996). Thioredoxin is an endogenous multifunctional protein with a redox-active disulfide/dithiol within the conserved active site sequence. It is also a scavenger of reactive oxygen species (ROS). Again Trx has not only anti-oxidant effect but also antiapoptotic effect (Turoczi et al., 2003). Therefore sildenafil mediated induction of Trx is promising. Sildenafil along with Trx also activate another stress protein, heme oxygenase-1 (HO-1/HSP 32). HO-1 catalyzes breakdown of the protoporphyrin ring, producing biliverdin, carbon monoxide (CO), and free ferrous iron (Okuyama et al., 2004). To this date, three isoforms of heme oxygenase have been identified (Maines, 1997). Among them, HO-1, unlike the other two (HO-2 and HO-3) shows limited expression under normal situations and is induced by a variety of physiological stimuli (McCoubrey et al., 1997; Elbirt and Bonkovsky, 1999; Choi and Alam, 1996). Transcriptional control of HO-1 is mediated by multiple factors including NFκB and AP-1. Evidence has been accumulating to suggest that HO-1 induction is an adaptive response to cellular stresses (McCoubrey et al., 1997; Elbirt and Bonkovsky, 1999; Choi and Alam, 1996; Maulik et al., 1996). Recent data indicate the involvement of CO in angiogenesis. Studies demonstrated the addition of CROME (CO-releasing molecule) or induction of HO-1 by hemin resulted in a three fold elevation in CO production in culture medium and was associated with a 30% increase in VEGF synthesis. Use of SnPP-IX prevented the induction of CO generation and inhibited the VEGF-mediated angiogenic activity of endothelial cells by the inhibition of cell proliferation (by 26%), migration (by 46%), formation of tuborogenesis on Matrigel (by 48%), and led to increased capillary sprouting (Jozkowicz et al., 2003).

Sildenafil mediated increased VEGF system and Angiopoietin system clearly documents its angiogenic property. This property may be due to the induction of Trx, HO-1, VEGF as well as Ang-1 which was abolished by the addition of SnPP (Fig. 6). Low dose sildenafil mediated activation of HCAEC tuborogenesis holds great promise to reduce tissue pathology through manipulation of Trx, HO-1, VEGF/Ang-1 expression using genetic or pharmacological strategies. For the first time our present data also suggests HO-1 mediated regulation of Ang-1 in sildenafil treated HCAEC. Application of SnPP along with sildenafil reduced significant protein expression of VEGF along with Ang-1 followed by the inhibition of tuborogenesis. In our present study sildenafil induced angiogenic response

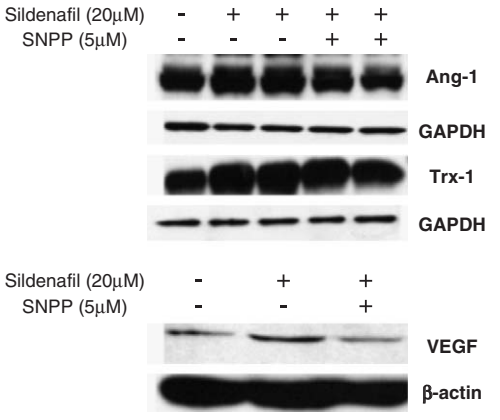

Fig. 5. Representative Western blot showing the effect of SnPP (5 μM) on the expression of Trx-1, Ang-1, VEGF in HCAEC. GAPDH and β-actin were used as loading controls.

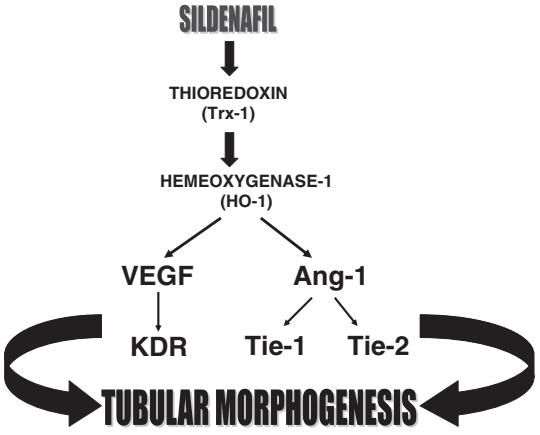

Fig. 6. Flow diagram showing the mechanism of sildenafil mediated tuborogenesis.

depends significantly upon VEGF receptor, KDR as well as angiopoietin receptors Tie-1 and Tie-2. They are tyrosine kinase receptors and responsible for cellular signaling triggered by VEGF, Ang-1 and 2. However, the other tyrosine kinase receptor of VEGF, Flt-1 was unaffected by sildenafil. Again, significant induction of VEGF, KDR, Ang-1, Tie-1 and Tie-2 was observed with 20 μM compared to 10 μM of sildenafil treatment. The tube formation was observed with even 1 μM of sildenafil however more complete ring-like structures were visible with 10–20 μM of sildenafil. These results strongly document the angiogenic effect of sildenafil. However there have been no systematic mechanistic studies with sildenafil mediated angiogenesis. Several studies have provided new insights into the signaling mechanisms of cardioprotection induced by sildenafil which include expression of nitric oxide synthases (NOS), accumulation of cGMP, activation of kinases (such as PKC and ERK) and opening of mitochondrial kATP channels (Jozkowicz et al., 2003).

5. Conclusion

Interestingly, our results indicate the induction of well-studied redox regulated proteins Trx-1, HO-1, VEGF and Ang-1 in human coronary arteriolar endothelial cells when exposed to low dose of sildenafil. The findings of the present in vitro study by sildenafil will offer a potentially attractive approach in vivo to increase endogenous protective molecules in the ischemic myocardium that may ultimately translate into novel therapeutic interventions.

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