Effect of Inducers of Heme Oxygenase-1 on Eicosanoids, cAMP and cGMP levels in Rat Endothelial Cells

M.T.Abdel-Aziz, M.F. Al-Asmar*, H. Atta, O. Shaker, H.H. Fouad, H. Hosni, N. Kamal, L. Rashed
Department of Medical Biochemistry, Faculty of Medicine, Cairo University
Department of Medical Biochemistry, Faculty of Medicine, Ain Shams University, Egypt

ABSTRACT

The present study was carried out to compare the effects of heme oxygenase -1 gene transfer into rat endothelial cells on cell proliferation, and on levels of cGMP, cAMP, PGI_2 and TXB_2. The transduced cells exhibited enhanced ability to express HO-1 mRNA as detected by RT- PCR. The proliferation of the transduced cells significantly increased compared to non-transduced cells. The cGMP and cAMP levels in transduced cells increased by 6.29 and 1.25 folds respectively, compared to non-transduced cells. Furthermore, the levels of PGI_2 and TXB_2 in transduced cells were decreased by 17.67 and 8.52 folds, respectively, relative to that in non-transduced cells. In this study, induction of HO-1 gene by injurious stimuli such as snake venom metalloproteinase with disintegrin like activity (SVMP), endotoxin (lipopolysaccharide, LPS) and H_2O_2 was studied in relation to cell proliferaion as well as levels of cGMP, cAMP, PGI_2 and TXB_2. SVMP increased the levels of cGMP and cAMP by 8.88 and 9.37 folds respectively, in transduced cells compared to non-transduced cells. Furthermore, SVMP decreased the levels of PGI_2 and TXB_2 by 26.85 and 13.95 folds respectively, in transduced cells compared to non-transduced cells. LPS increased the levels of cGMP and cAMP by 8.88 and 4.68 folds respectively, in transduced cells. LPS decreased the levels of PGI_2 and TXB_2 by 53.02 and 27.77 respectively, in transduced cells as compared to non-transduced cells. H_2O_2 increased the levels of cGMP and cAMP by 47.77 and 6.25 folds, respectively, in transduced cells. Furthermore, H_2O_2 decreased the levels of PGI_2 and TXB_2 by 183.3 and 2.52 folds respectively, in transduced cells as compared to non-transduced cells. Moreover, H_2O_2 decreased cell proliferation significantly in both transduced and non-transduced cells. SVMP and LPS had no effect on cell proliferation in both transduced and non-transduced cells. Although, SVMP, LPS and H_2O_2 had no effects on PGI_2, cGMP and cAMP levels in non transduced cells, these injurious agents had an enhancing effect on TXB_2 levels in these cells (increase by 2.66, 1.38 and 1.83 folds, respectively).

RIASSUNTO

Effetto degli induttori della eme ossigenasi-1 sui livelli di eicosanoidi, cAMP e cGMP nelle cellule endoteliali di ratto

Il presente studio è stato effettuato al fine di comparare gli effetti sulla proliferazione cellulare, e sui livelli di cGMP, cAMP, PGI2 and TXB2, del trasferimento in cellule endoteliali di ratto del gene della eme ossigenasi-1. Le cellule transdotte mostravano una aumentata capacità di esprimere HO-1 mRNA evidenziabile mediante RT- PCR. La proliferazione delle cellule transdotte era significativamente aumentata rispetto a quelle non-transdotte. I livelli di cGMP e di cAMP nelle cellule transdotte risultavano aumentati di 6.29 e 1.25 volte rispettivamente in confronto alle non-transdotte mentre, nel medesimo confronto, i livelli cellulari di PGI2 e di TXB2 risultavano ridotti di 17.67 e 8.52 volte, rispettivamente. In questo lavoro è stata studiata l'induzione del gene della HO-1 mediante stimolazioni nooocive come quelle da metalloproteinasi di veleno di serpente con attività disintegrino-simile (SVMP), da endotossina (lipopoliaccaride, LPS) e da H2O2, in rapporto alla proliferazione cellulare come pure ai livelli di cGMP, cAMP, PGI2 e TXB2. SVMP provocava un aumento dei livelli di cGMP e cAMP, pari a 8.88 e 9.37 volte rispettivamente. Nelle cellule transdotte rispetto a quelle non-transdotte. Inoltre SVMP provocava una diminuzione dei livelli di PGI2 e di TXB2, di 26.85 e 13.95 volte rispettivamente, nelle cellule transdotte rispetto a quelle non-transdotte. LPS provocava un aumento dei livelli di cGMP and cAMP (8.88 e 4.68 volte, rispettivamente) ed una diminuzione dei livelli di PGI2 e TXB2 (53.02 e 27.77 volte rispettivamente) nelle cellule trasdotte rispetto alle non-transdotte. H2O2 provocava un aumento di dei livelli di cGMP e cAMP (47.77 e 6.25 volte, rispettivamente) ed una diminuzione dei livelli di PGI2 e TXB2 (183.3 e 2.52 volte rispettivamente) nelle cellule transdotte rispetto alle non-transdotte. Inoltre, H2O2 diminuiva significativamente la proliferazione cellulare sia delle cellule transdotte sia nelle non-transdotte, mentre SVMP e LPS non avevano effetto sulla proliferazione cellulare di entrambe le cellule.Nonostante che SVMP, LPS e H2O2 non avessere alcun effetto sui livelli di PGI2, cGMP e cAMP nelle cellule non-transdotte, questi agenti nocivi aumentavano i livelli di TXB2 in queste cellule (2.66, 1.38 and 1.83 volte, rispettivamente).

INTRODUCTION

Heme oxygenase (HO-1) controls the initial and rate limiting step in heme catabolism. The enzyme cleaves heme to biliverdin, which is subsequently cleaved to bilirubin by biliverdin reductase. Iron is released when the heme ring is opened, while carbon monoxide (CO) is liberated. So, this enzyme down-regulates all cellular hemoproteins (1). The heme molecule plays a central role in diverse biological processes as the prosthetic moiety of heme proteins is involved in cell respiration, energy generation (cytochromes of ETS), oxidative biotransformation (Cytochrome P_{450}), growth differentiation processes and the generation of the inflammatory mediators such as eicosanoids by affecting cyclooxygenase (COX) and nitric oxide synthase (NOS) (2,3). So, HO-1 down-regulates NO and eicosanoids and has a prominent anti-inflammatory effects.

To date, three HO isoforms (HO-1, HO-2 and HO-3) have been identified (4). HO-1 is a 32 kDa heat shock protein that is inducible by numerous noxious stimuli (5). HO-2 is a constitiutively synthesized 36-kDa protein that is abundant in brain and testis. HO-3 is related to HO-2 but is the product of a different gene and its ability to catalyze heme degradation is lower than that of HO-2 (6,7).

HO-1 enzyme belongs to a class of macromolecules known as stress proteins, which are responsive to various types of cellular injuries. Induction of HO-1 is thought to be of considerable importance in the initiation of cellular protective mechanisms following exposure to various types of cell stressful stimuli such as: extravasation of heme, oxidative stress inducing agents, endotoxins, hyperthermic shock and inflammatory cytokines (8,9).

HO-1 gene initiates numerous protective mechanisms against hypoxia, ischemia and other cell stressful stimuli. Increased HO-1 activity enables the removal of heme, a lipid soluble transmissible form of a potent pro-oxidant iron. HO-1 induction leads to the release of CO that has numerous biological effects that mimics NO such as: vasodilator effects on blood vessels, i.e. protection against cellular ischemia and ensures adequate tissue perfusion, induction of soluble guanylate cyclase with subsequent increase in cGMP which acts as neural messenger, regulation of Na/K ATPase pump and decrease of adhesion molecules (4). Furthermore, HO-1 induction leads to the release of bilirubin which has a pronounced anti-oxidant, anticomplement and angiogenic properties. Moreover, HO-1 gene induction leads to activation of hypoxia inducible factor genes (HIF-1 genes) which enhances growth factors that are involved in endothelial cells proliferation and cFos proto-oncogene activation (10). HO-1 gene overexpression leads to upregulation of ferritin that sequesters iron, down regulation of heme dependent enzymes such as COX and NOS, inhibition of platelet aggregation through effects on COX, inhibition of monocytes transmigration induced by LDL and endothelial cell proliferation, activation and angiogenesis via ETS effects (11).

The aim of this work is to study rat lung microvessel endothelial cells (RLMV) cell proliferation as well as the levels of cGMP, cAMP, PGI_2 and TXB_2 in the media of cultured transduced with HO-1 gene and non transduced RLMV cells. Moreover, induction of HO-1 gene by injurious stimuli such as SVMP, LPS and H_2O_2 was studied in transduced and non-transduced cells as regards proliferation as well as the levels of cGMP, cAMP, PGI_2 and TXB_2 in cell culture media.

MATERIALS AND METHODS

Rat lung microvessel endothelial cells (RLMV) transduced and non-tranduced with HO-1 gene via retroviral packaging cell line PA317 (American Type Culture Collection) were a generous gift from Prof. Dr. N. Abraham, New York Medical College, Valhalla, NY, USA. Human HO-1 gene was transferred to RLMV by a retroviral vector LSN-hHO.

Cell culture conditions

Non-transduced and transduced RLMV were grown in DMEM (GIBCO-BRL, Grand Island, NY) supplemented with 10 % heat inactivated FBS (GIBCO-BRL), 100 U / mL streptomycin (GIBCO-BRL). All cells were incubated at 37 in 5 % CO_2 humidified atmosphere and were maintained at subconfluency. Cells were subcultured using trypsin - EDTA (GIBCO-BRL).

Reagents

Endotoxin (lipopolysaccharides, LPS) and H_2O_2 were obtained from Sigma Chemical (St. Louis, MO). SVMP was a generous gift from Prof. Dr. M. F. Al-Asmar, Faculty of Medicine, Ain Shams University. Inducers were added to the tissue culture media at a concentrations of 100 pg/mL for LPS, 300 μmol/mL for H_2O_2 and 1 μg/mL for SVMP for a period of 24 hours.

Cells were separated by centrifugation for RNA extraction, the supernatant was used for estimation of total proteins (12), cGMP, cAMP, PGI_2 and TXB_2 levels.

RNA extraction

Total RNA was extracted from the cells using SV total RNA isolation system provided from Promega, Madison, WI, USA.

RT - PCR for heme oxygenase -1 detection

Two oligonucleotide primers were prepared to amplify a 555 bp stretch of human HO-1 gene with the following sequence: (sense) 5' CAG GCA GAG AAT GCT GAG TTC 3' and (antisense) 5' GAT GTT GAG CAG GAA CGC AGT 3' (Yoshida et al., 1988). RNA was reverse transcribed using 12.5 μl oligonucleotide (dT)18 primer (final concentration 0.2 μMol) and was denatured at 70°C for 2 minutes. The denatured RNA was placed on ice and 6.5 μl of reverse transcription mixture containing 50 mM KCl, 50 mM Tris HCl, pH 8.3, 0.5 mM of dNTPs, 3 mM $MgCl_2$, 1U/μl

RNAse inhibitor and 200 Units of MMLV reverse transcriptase were added. Then, the reaction tube was placed at 42°C for 1 hour followed by heating to 92°C to stop the reaction then placed on ice. The PCR reaction was performed by adding to the reverse transcription tube, the PCR mix to a final volume of 100 µl. The PCR mix contained 10 mmol/l Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin, 250 µM dNTP mix, 2.5 U Taq polymerase and 100 µM of each primer. The reaction mixture was then subjected to 40 cycles of PCR amplification as follows: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. After the last cycle a final extension at 72°C for 10 min was done.

Agarose gel electrophoresis

PCR product was electrophoresed on 1.5% agarose gel with size marker and were UV visualized by ethidium bromide.

Measurement of cell proliferation (viability)

Cell viability was assayed using a colorimetric method according to the manufacturer recommendations (Cell titre 96 AQ_{ueous} one solution cell proliferation assay, Promega, Madison, WI) which is more sensitive, less time consuming, and requires fewer cells than more traditional methods such as trypan blue exclusion. The cells were placed in 96 - well plates and treated with various HO-1 inducers as described above. At the indicated times, to each well, 20 ul of cell titre 96 AQ_{ueous} One solution reagent was added, which contained a tetrazolium salt (MTS) and an electron coupling reagent (PMS).The cells were then incubated at 37°C for 1.5 hrs. The amount of tetrazolium reduced to a chromophore derivative by viable cells was quantified spectrophotometrically at 490 nm using microplate reader. There is a linear relationship between the formazan generated and the number of viable cells present. The number of cells surviving was expressed as the ratio of the optical density at 490 nm (OD_{490}) of the cells treated with HO-1 inducers over the OD_{490} of control cells. Experiments were performed with six replicates.

Assay of cGMP, cAMP, PGI_2 and TXB_2

Cellular concentrations of the previous mentioned parameters were assayed in the supernatant of culture medium using ELISA kits for these parameters obtained from Quantikine, R&D system, Minneapolis, MN, USA according to the manufacturer description.

RESULTS

Results are summarised in the following tables and figure.

DISCUSSION

Retroviruses can be used to express target genes in host cells due to their characteristic incorporoation of the gene into the host cell chromosomes. Indeed several cell lines that specifically express certain target genes in host cells with a long term expression have been developed (2,13). In the present work, a retroviral vector (LSN - hHO) for gene transfer of HO-1 was constructed and used to transfect rat lung microvessel endothelial cells (RLMV / hHO) (4). In the present study, the specific hHO-1 mRNA transcription in the transduced cells was confirmed by RT - PCR. Furthermore, the proliferation of the transduced cells was significantly increased as compared to non transduced cells. The cGMP and cAMP levels in the culture media of transfected cells increased relative to that in non-transfected cells. Moreover, levels of PGI2 and TXB2 decreased in transduced cells as compared to non transduced cells. These results suggest that overexpression of hHO-1 gene, may contribute to the elevated cellular levels of cGMP and cAMP. Yang et al.,(4) reported similar findings. They stated that over expression of hHO-1 gene in rat endothelial cells was associated with an elevation of cGMP. Durante et al., (14) stated that cAMP induces HO-1 gene expression and CO production in vascular smooth muscle cells. The capacity of cAMP to induce the synthesis of guanylate cyclase -stimulated CO from smooth muscle cells may represent a novel mechanism by which this nucleotide regulates vascular tone. The biological

Table 1
Levels of cGMP, cAMP, PGI_2 and TXB_2 after induction of HO-1 gene in transduced cells compared to non-transduced cells

Parameters	Non-transduced Control cells	Transduced cells			
		Blank cells Not-induced	SVMP Induction	Endotoxin Induction	H_2O_2 Induction
PGI_2 pg/mg protein	14728.72	833.33	548.38	277.77	80.35
TXB_2 pg/mg protein	1800	211.11	129.03	64.81	714.28
cGMP pmol/mg protein	1.350	8.5	12	12	64.5
cAMP pmol/mg protein	0.016	0.02	0.15	0.075	0.1

Table 2
Levels of cGMP, cAMP PGI_2 and TXB_2 after induction of HO-1 gene in non-transduced cells

Parameters	Non-transduced Control cells	Induced Non-transduced cells		
		SVMP Induction	Endotoxin Induction	H_2O_2 Induction
PGI_2 pg/mg protein	14728.72	13571.4	14074	13946.4
TXB_2 pg/mg protein	1800	4800	2500	3300
cGMP pmol/mg protein	1.350	0.9	0.9	1.3
cAMP pmol/mg protein	0.016	0.014	0.017	0.019

Table 3
Effect of heme oxygenase inducers on proliferation of transduced rat lung microvessel endothelial cells

	Control Non-transduced Not induced	Induced -Transduced Cells			
		Transduced Not Induced cells	SVMP Induction	Endotoxin Induction	H_2O_2 induction
Cell Proliferation OD_{490}	0.0187 ± 0.004	0.027± 0.001 P < 0.0001	0.022 ± 0.007 P N.S.	0.024 ± 0.006 P N.S.	0.009 ± 0.002 P < 0.0001

Table 4
Effect of heme oxygenase inducers on proliferatio

	Control Non-transduced Not induced	Induced Non - Transduced Cells		
		SVMP Induction	Endotoxin Induction	H_2O_2 induction
Cell Proliferation OD_{490}	0.0187 ± 0.004	0.016 ± 0.002 P N.S.	0.017 ± 0.003 P N.S.	0.002 ± 0.001 P < 0.0001

significance of elevated cGMP levels after hHO-1 gene transduction was elucidated. Carbon monoxide (CO), a by-product derived from HO reaction shares many biological properties of NO such as vasodilatation, decreasing adhesion molecules levels and increasing cGMP levels that is considered a putative neural messenger and a regulator of Na^+/K^+ ATPase pump (10,15).

On the other hand, levels of PGI_2 and TXB_2 in transduced cells exhibited significant decrease as compared to their levels in non-transduced cells. Cyclo-oxygenase is a hemoprotein and thromboxane synthase is cytochrome P_{450} like enzyme requiring a heme iron - centered oxygen attack of the prostaglandin endoperoxide substrate (PGH_2) for subsequent thromboxane A_2 (TXA_2) synthesis. Sessa et al., (16) proved that the activity and levels of P_{450} enzymes can be manipulated by decreasing heme availability. Over expression of hHO-1 gene leads to enhanced degradation and down regulation of all hemoproteins. Reduction of PGI_2 and TXB_2 levels, explain the anti-inflammatory and anti-thrombogenic effects of hHO. Wagner et al., (17) reported that up-regulation of hHO-1 gene expression leads to inhibition of platelet aggregation in vascular smooth muscle cells and this fact could be explained by the down regulatory effects of HO-1 gene on thromboxane synthase. Goodman et al., (18), Abraham et al., (19) and Botros et al., (20) stated that overexpression of HO-1 in microvessel endothelial cells and in rat kidney cells leads to a significant reduction of COX1 and COX2 with subsequent decrease in PGI_2, TXB_2, PGE_2 and 6-Keto PGF1a. Such effects may play an important role in the regulation of vascular tone, blood pressure and renal hemodynamics.

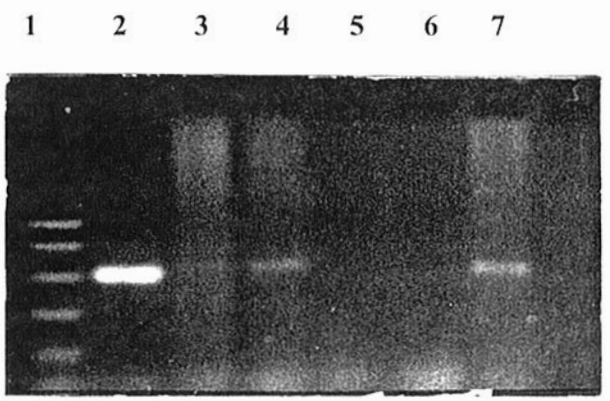

Figure 1
Shows 1.5 % agarose gel electrophoresis of HO-1 PCR products of transduced and non-transduced cells together with the presence of different HO-1 inducers.
Lane 1: PCR marker (1000, 750, 500, 300, 150, 50).
Lane 2 : Positive Control (500 bp).
Lane 3: Non- transduced control endothelial cells.
Lane 4: HO-1 transduced endothelial cells.
Lane 5: HO-1 transduced endothelial cells in the presence of LPS.
Lane 6: HO-1 transduced endothelial cells in the presence of SVMP.
Lane 7: HO-1 transduced endothelial cells in the presence of H_2O_2.

In mammalian cells, a variety of stress inducers such as endotoxin, H_2O_2, oxidized lipoproteins, heme and non heme mediated oxidant injury, can cause robust up-regulation of HO-1 activity. The induction of HO-1 is thought to be an adaptive response that offers cytoprotection to cells / tissues against oxidant stress (21, 22). In the present work, induction of HO-1 by LPS, SVMP and H_2O_2 in both transduced and non-transduced cells was studied. The present results showed that after induction of HO-1 by SVMP, LPS and H_2O_2, there was a significant increase in cAMP and cGMP in transduced cells as compared to non-transduced cells, whereas levels of PGI_2 and TXB_2 exhibited significant decrease in transduced cells as compared to non-transduced cells. These observations demonstrate that the various noxious inducers used in our experiment lead to HO-1 gene up-regulation with subsequent modulatory effects on eicosanoids, cAMP and cGMP. In conclusion, chemical and /or pharmacological manipulation of HO-1 to further enhance or suppress the gene may have therapeutic potential as HO-1 protein has diverse biological effects in all cellular aspects.

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