Differential regulation of cardiac heme oxygenase-1 and vascular endothelial growth factor mRNA expressions by hemin, heavy metals, heat shock and anoxia

Rodrigo Eyssen-Hernandez, Annie Ladoux, Christian Frelin*

Institut de Pharmacologie Moléculaire et Cellulaire du CNRS, Université de Nice-Sophia Antipolis, 660 Route des Lucioles, 06560 Valbonne, France

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Abstract Increasing attention has been paid to the effects of hypoxia, heavy metals and heat shocks on gene expression and to the similarities in their actions. This paper compares mRNA levels of two putative hypoxia, heavy metal and heat shock sensitive genes: heme oxygenase-1 (HO-1) and vascular endothelial growth factor (VEGF) in myocyte-enriched cultures of neonatal rat heart cells. HO-1 mRNA expression is stimulated by hemin, Cd^{2+}, Co^{2+} and heat shocks but not by Ni^{2+} or Mn^{2+}. It is stimulated by long (13 h) but not by short (4 h) periods of anoxia. Conversely, VEGF mRNA expression is stimulated by short as well as long periods of anoxia, by Cd^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} but not by hemin or heat shocks. The results suggest that heavy metals, anoxia and heat shocks exert their effects on VEGF and HO-1 mRNA expression through separate though potentially overlapping mechanisms. Increased expressions of HO-1 and VEGF may be both cardioprotective under hypoxic/ischemic conditions.

Key words: Hypoxia; Vascular endothelial growth factor; Heme oxygenase; Cobalt; Cadmium; Heat shock

1. Introduction

Increasing evidence suggests that mammalian cells sense their oxygen environment and adapt to it by changing their pattern of gene expression. Examples of hypoxia regulated genes are erythropoietin [1], vascular endothelial growth factor (VEGF) [2,3], enzymes of the glycolytic pathway [4–6], heme oxygenase-1 (HO-1) [7,8], heat shock proteins [9] and tyrosine hydroxylase [10].

Among these hypoxia sensitive genes, HO-1 and VEGF are expressed in cardiac tissues [2,3,7,11] and are of particular importance for protecting hearts against hypoxia. HO-1 (EC 1.14.99.3) catalyses the initial reaction in heme catabolism: the oxidative cleavage of the a-mesocarbon bridge of b-type heme molecules to yield equimolar quantities of biliverdin IXa, carbon monoxide and iron. Carbon monoxide is a potent activator of soluble guanylate cyclase [12] and vasodilator substance [13]. VEGF is a potent and specific endothelial cell mitogen. It probably plays an important role in the formation of tumor associated vasculature [14] and has been shown to induce collateral vessel development in a rabbit model of hind limb ischemia [15].

Circumstantial evidence suggests that HO-1 and VEGF expressions are regulated by similar if not identical mechanisms. In non-cardiac tissues, HO-1 is induced in different situations of stress such as oxidative damage, heat shock and UVA radiation [16], by heavy metals such as Cd^{2+} and Co^{2+} and by its substrate hemin [17,18]. It is induced by hypoxia in rat hearts [7] and Chinese hamster ovary cells [8]. Conversely, cardiac VEGF expression is stimulated by hypoxia and Co^{2+} [2,3,11,19].

To analyze further the mechanisms involved in these regulations, this study compares VEGF and HO-1 mRNA expressions by myocyte-enriched cultures of new born rat heart cells exposed to anoxia, heat shocks and to heavy metals.

2. Materials and methods

2.1. Chemicals

All enzymes and tissue culture media were from Gibco BRL unless specified. Radioactive materials were from ICN. Fetal calf serum was from Boehringer and horse serum from D. Dutcher (Strasbourg, France). All chemicals were from Sigma.

2.2. Cell culture

Myocyte-enriched cultures were prepared from new born rats as previously described [20]. Cells were grown on gelatin-coated dishes for 3 days in Ham's F12 medium supplemented with 2 mM glutamine, 10% heat inactivated fetal bovine serum and 10% heat inactivated horse serum. After washing, cells were further incubated for 24 h into a 2% serum culture medium and then exposed for 4 h (unless otherwise indicated) to different experimental conditions. A low serum condition was used to decrease the endogenous expression of VEGF mRNAs due to activation of serum responsive elements observed when 10% serum was used [3]. Anoxic conditions were induced by incubating cells (in a 10 mM HEPES-buffered and bicarbonate-free medium) for 4 h in an air tight container flushed with pure N_2 [3]. A heat shock was produced by exposing cells to 41°C for 4 h.

cGMP measurements were performed using the Amersham assay kit according the instructions of the manufacturer. Cells were exposed to 100 μM hemin or to 41°C for 4 h in the presence of 0.1 mM isobutyl methyl xanthine.

2.3. Northern blots

Total RNA was extracted according to Chomczynski and Sacchi [21]. 10 μg of total RNA were fractionated by denaturing electrophoresis on 1.2% agarose formaldehyde gels and transferred to Nytran membranes (Schleicher and Schuell) prior to hybridisation with selected probes. The rat HO-1 probe was a 159 base pair fragment comprised between nucleotides 436 and 595 of the coding sequence. It was generated by polymerase chain reaction from mRNA isolated from Co^{2+} treated cardiac myocytes [22]. The rat VEGF probe was a 350 base pair fragment comprised between nucleotides 168 and 517 of rat VEGF 164 [23]. PCR products were subcloned into the *SmaI* site of bluescript SK(−) plasmids (Stratagene) and sequenced by the dideoxy terminator sequencing kit (Applied Biosystem). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a gift of Dr. F. Moreau-Gachelin (Paris). cDNA probes were labelled using [^{32}P]dCTP and the Prime-a-Gene labeling system (Promega). Autoradiography was performed with intensifying screens at −80°C using Kodak X-Omat AR films. Relative amounts of radiolabelled cDNA that hybridized to the blots were quantitated using the NIH image software and normalized to GAPDH mRNA levels to control for loading errors. VEGF/GAPDH and HO-1/GAPDH signal ratio were set at an arbitrary value of 1 in control experiments.

Means ± S.E.M. ($n > 3$) are indicated.

*Corresponding author. Fax: (33) 93 95 77 08.

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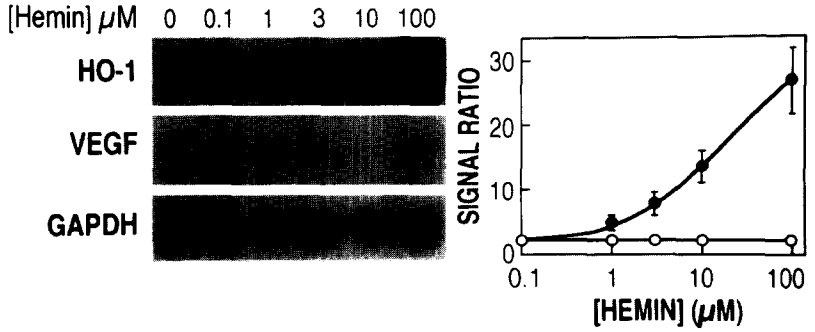

Fig. 1. Hemin stimulates HO-1 mRNA expression. Left: Dose dependent accumulation of HO-1 mRNA in hemin stimulated cells. Cardiomyocytes were incubated for 4 h with the indicated concentrations of hemin and Northern blots hybridized with HO-1, VEGF and GAPDH probes as indicated. Right: Dose response curve for hemin action on normalized HO-1 (●) and VEGF (○) hybridization signals.

3. Results

Cardiomyocyte-enriched cultures of new born rat heart cells were prepared and exposed to different experimental conditions known to alter HO-1 or VEGF gene expressions in other cell types. RNA was extracted and analyzed by Northern blots. All hybridization signals were normalized to that of GAPDH.

Hemin, the substrate of HO-1, is known to induce HO-1 expression in other tissues [18]. Unstimulated rat cardiomyocytes expressed low levels of HO-1 and VEGF mRNAs. Exposing cardiac cells to hemin induced a large (up to 27 fold) increase in HO-1 mRNA levels (Figure 1). Induction was already detected after 1 h of exposure to 100 μM hemin and was maximum at 6 h (data not shown). The action of hemin developed at concentrations between 1 and 100 μM. Under identical conditions, hemin exerted no significant action on VEGF mRNA levels (Fig. 1).

Neonatal cardiomyocytes mainly rely on glycolysis to maintain high intracellular ATP levels. They can be maintained under anoxic conditions for several hours without impairing spontaneous contractile activity [24]. Fig. 2 shows that a 4 h anoxia increased 5 fold VEGF mRNA levels. It had no significant influence on HO-1 mRNA levels. More prolonged anoxic periods increased both HO-1 and VEGF mRNA levels. In these series of experiments and after correcting for loading, anoxia increased the VEGF hybridization signal up to 8 fold. It increased the HO-1 hybridization signal only 2 fold. More prolonged anoxic periods led to erratic results and were associated with extensive cell damage. Thus, VEGF mRNA expression in response to anoxia appeared sooner and was more pronounced than that of HO-1.

We next compared the actions of heavy metals on HO-1 and VEGF mRNA expressions. Heavy metals such as Co^{2+}, Ni^{2+} and Mn^{2+} are thought to induce VEGF gene expression by activating hypoxia sensitive elements [2]. Cd^{2+} and Co^{2+} increase HO-1 gene expression via well identified metal responsive elements [25].

Fig. 3 shows that Co^{2+} induced increased expressions of both VEGF and HO-1 mRNAs. Its action on HO-1 mRNA expression was already detected after 1 h and reached a maximum at 2–4 h, similar to what has been observed with VEGF [11]. Dose response curves were, however, markedly different. A statistically significant action of Co^{2+} on VEGF mRNA expression was only observed at 30 μg/ml $CoCl_2$ (corresponding to 230 μM Co^{2+}) and the maximum stimulation observed was only 2 fold. Higher concentrations led to more erratic results. When data from different experiments were pooled, no statistically significant increase in VEGF mRNA expression was observed at 50 or 100 μg/ml $CoCl_2$. Fig. 3 shows that Co^{2+} increased HO-1 mRNA expression in a dose-dependent manner and that the maximum stimulation produced at 100

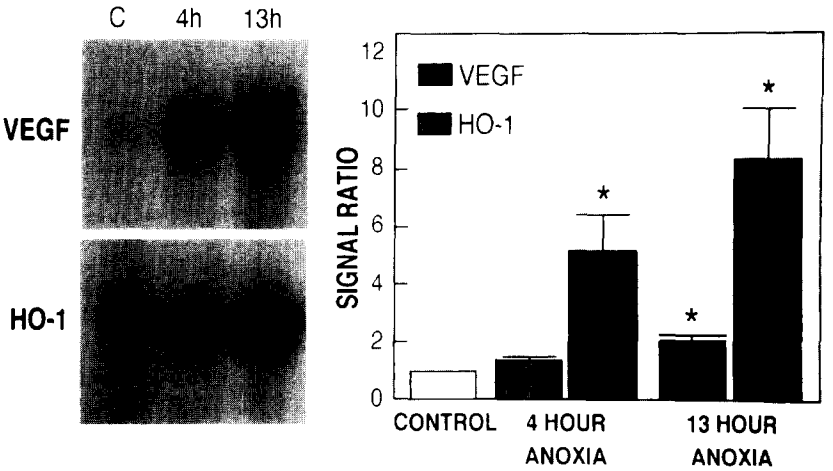

Fig. 2. Anoxia induces VEGF and HO-1 mRNA expressions. Left: Representative Northern blots showing the action of 4 h and 13 h anoxia on VEGF and HO-1 mRNA levels. Right: Normalized HO-1 and VEGF hybridization signals. *$P < 0.01$ as compared to controls.

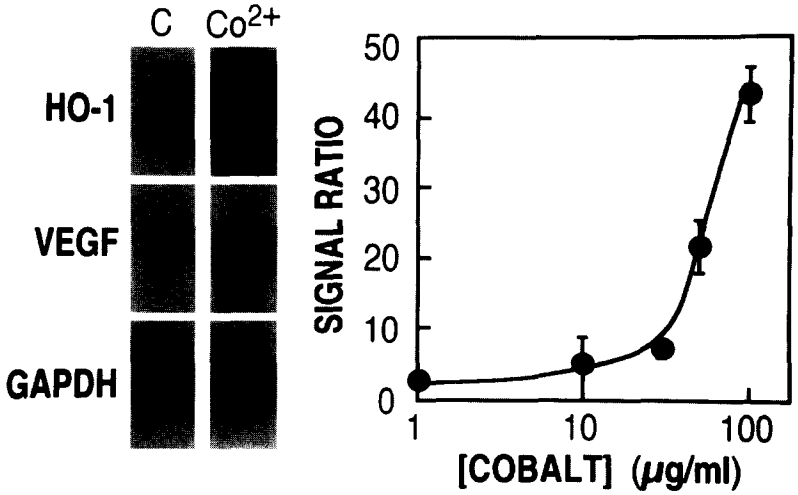

Fig. 3. Cobalt stimulates HO-1 mRNA expression. Left: Representative Northern blots showing the actions of 230 μM Co^{2+} on VEGF and HO-1 mRNAs. Right: Dose response curve for Co^{2+} action on HO-1 hybridization signal. Its action on the VEGF signal (2 fold increase) was too low to be represented on the same scale.

μg/ml reached 46 fold. This action of Co^{2+} was completely prevented by actinomycin D (5 μg/ml) and by cycloheximide (10 μg/ml) (data not shown). Thus, Co^{2+} was a strong inducer of HO-1 mRNA expression. It was a much weaker inducer of VEGF mRNA expression.

We next examined the action of Cd^{2+}, a potent inducer of HO-1 mRNA expression [18]. Fig. 4 shows that Cd^{2+} increased HO-1 mRNA levels in rat cardiomyocytes. Induction was already detected after 1 h and reached a maximum at 2–4 h. It developed at low micromolar concentrations (Fig. 4). In the same cells, Cd^{2+} also increased VEGF mRNA levels with a similar time course. VEGF mRNA expression was, however, less sensitive to the action of Cd^{2+} than that of HO-1 (Fig. 4). After normalization of the hybridization signal, Cd^{2+} increased HO-1 mRNA levels 10 fold. It increased VEGF mRNA levels only 4 fold.

Ni^{2+} and Mn^{2+} (100 μM) increased VEGF mRNA levels about 2 fold in cardiomyocytes [11]. They had no significant action on HO-1 mRNA expression (data not shown).

Fig. 5 shows that a heat shock increased HO-1 mRNA expression 4 fold. It had no action on VEGF mRNA levels.

HO-1 generates carbon monoxide, a potent agonist of soluble guanylate cyclase [12]. An obvious question raised by our experiments was whether induction of HO-1 mRNA expression led to cGMP formation. No effect of heat shock and hemin (100 μM) on cGMP levels was observed in cultured cardiomyocytes. We also observed that the cells used did not respond to 0.1 mM sodium nitroprusside by the formation of cGMP, hence indicating that they probably lack soluble guanylate cyclase activity.

4. Discussion

Expression of HO-1 mRNAs in cardiomyocytes is strongly enhanced by hemin, Co^{2+} and Cd^{2+}. It is moderately enhanced by hypoxia and heat shocks. This pattern of regulation is similar to that described in other cell types [8,16–18]. Expression of VEGF mRNAs is similar in many respects to that of HO-1 but differs substantially. VEGF mRNA expression is stimulated by hypoxia, Co^{2+} and Cd^{2+} but not by hemin or heat shocks.

Studies derived from the erythropoietin field suggest that Co^{2+} acts by replacing Fe^{2+} in the heme molecule associated with putative hypoxia sensitive transcription factors and by locking them into an active deoxy form [1]. The observation that Co^{2+} mimics the effects of hypoxia on VEGF mRNA

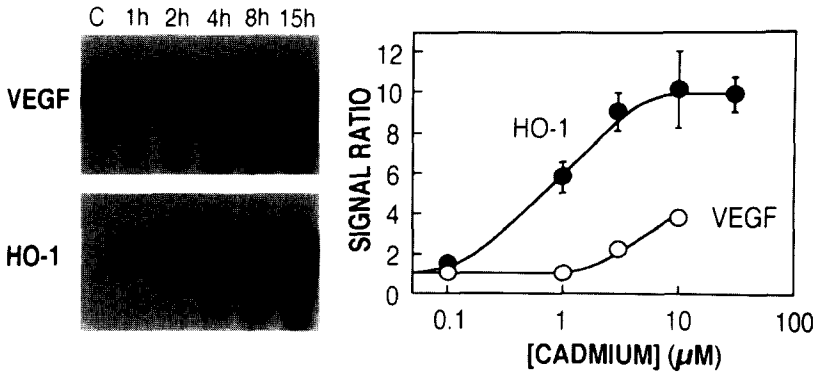

Fig. 4. Cadmium stimulates HO-1 and VEGF mRNA expressions: Left: Representative Northern blots showing the time course of action of 10 μM Cd^{2+} on VEGF and HO-1 mRNA expressions. Right: Dose response curves for Cd^{2+} action on normalized HO-1 (●) and VEGF (○) mRNA levels. Time of exposure to Cd^{2+} was 4 h.

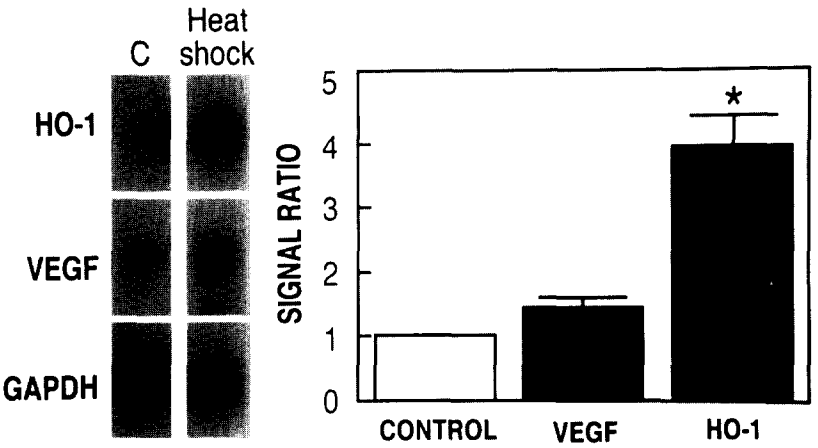

Fig. 5. Heat shock activates HO-1 but not VEGF mRNA expression: Left: Representative Northern blot showing the actions of 4 h exposures to 41°C on HO-1, VEGF and GAPDH mRNA expressions. Right: Normalized HO-1 and VEGF hybridization signals. $*P < 0.01$ as compared to controls.

expression has been taken as strong evidence in favor of the hypothesis that an oxygen sensor controls VEGF gene expression in a manner independent of the energetic state of the cells [2,11]. The difficulty with this hypothesis is that the specificity of Co^{2+} has not been demonstrated. A first action of heavy metals could be to block L-type Ca^{2+} channels [26]. The observations that VEGF [11] and HO-1 (data not shown) mRNA expressions are not affected by 0.1 μM PN 200-110, a potent blocker of L-type Ca^{2+} channels suggest that actions of heavy metals reported here were unlikely associated to an impaired excitability. Another action of heavy metals is to activate gene transcription by acting via hypoxia sensitive elements [27] (that are present in the VEGF gene but not in the HO-1 gene) or via metal responsive elements that have been characterized in the metallothionein [28] and HO-1 genes [25]. The observations that Cd^{2+} and Co^{2+} increase HO-1 mRNA expression (Figs. 3 and 4) indicate that heavy metals enter cardiomyocytes via yet unidentified pathways and are able to activate directly or indirectly metal responsive elements in the HO-1 gene promoter. Analysis of the VEGF gene sequence shows the presence both of putative metal responsive elements (homologous to those present in the HO-1 gene) and of functional hypoxia sensitive elements [27]. An obvious question is therefore whether heavy metals induce VEGF mRNA expression by activating metal responsive elements or hypoxia sensitive elements. The close similarities between heavy metal actions on VEGF and HO-1 mRNA expressions suggest that they act via metal responsive elements rather than via hypoxia sensitive elements. Although further analysis is required to ascertain that putative metal responsive elements present in the VEGF gene are functional, this hypothesis is consistent with recent results showing that in human retinal pigment cells Co^{2+} increases VEGF mRNA expression by a mechanism distinct from hypoxia [29].

Expression of both VEGF and HO-1 mRNAs are stimulated in response to anoxia but with different sensitivities. (i) Expression of HO-1 mRNAs is delayed by several hours as compared to that of VEGF. (ii) Long periods of anoxia induce larger increases in VEGF mRNA levels (8 fold) than of HO-1 mRNA levels (2 fold) (Fig. 2). These could suggest anoxia acts by more than one type of mechanism. VEGF expression is thought to be determined by an oxygen sensor, independent of the energetic state of the cells [2]. Conversely, HO-1 expression by human skin fibroblasts is thought to be determined by the availability of cellular glutathione [16]. It should be noted, however, that in isolated perfused hearts, induction of VEGF mRNA expression by hypoxia is slowed down when contractile activity is arrested with tetrodotoxin [19]. This suggests that energy dependent mechanisms also determine VEGF mRNA expression at least in intact hearts.

HO-1 is considered as a heat shock protein [18,30,31]. In agreement, HO-1 mRNA expression is induced by hyperthermia in isolated rat hearts [32] and by heat shocks in cultured neonatal cardiomyocytes (Fig. 5). Increasing evidence suggests that hypoxia induces the expression of heat shock proteins and that hypoxia and heat shock act via similar if not identical mechanisms [9,33]. Our results show that this is probably not true in cardiac cells. HO-1 mRNA expression is clearly increased after a heat shock; that of VEGF is not (Fig. 5).

Taken together, these results stress the point that although heavy metals, hypoxia and heat shocks control the expression of similar sets of genes, they may act through separate, though potentially overlapping pathways.

Increased expression of both VEGF and HO-1 may be an important adaptative mechanism to improve blood flow under chronic hypoxic/ischemic conditions. VEGF is a potent angiogenic factor that induces the formation of neovessels in ischemic states [14,15]. HO-1 on the other hand generates bile pigments that act as radical scavengers [34]. It also produces carbon monoxide, a potent agonist of soluble guanylyl cyclase and vasodilator substance [12,13]. It is of interest that hyperthermia which increases HO-1 expression also induces the formation of cGMP in intact hearts [32]. This is not true in cultured cells. All treatments that induce HO-1 mRNA expression in cultured cardiomyocytes do not increase cellular GMP levels probably because cells lack soluble guanylate cyclase. It could be that in intact hearts, carbon monoxide produced by HO-1 mainly acts in a paracrine manner to activate soluble guanylate cyclase in the coronary vasculature.

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