Induction by prostaglandin A_1 of haem oxygenase in myoblastic cells: an effect independent of expression of the 70 kDa heat shock protein

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Prostaglandins of the A type (PGA) induce the synthesis of 70 kDa heat shock proteins (hsp70) in a large variety of mammalian cells. Induction of hsp70 has been associated with a cytoprotective effect of PGA_1 after virus infection or thermal injury. In the present report we provide evidence that, in murine myoblasts, PGA_1 is not able to induce hsp70 expression, whereas it increases the synthesis of the constitutive protein, hsc70, and dramatically induces the synthesis of a 32 kDa protein (p32). The p32 protein has been identified as haem oxygenase. PGA_1 acts at the transcriptional level by inducing haem oxygenase mRNA synthesis, and the signal for induction appears to be associated with decreased intracellular GSH levels. Haem oxygenase, a low-molecular-mass stress protein induced in mammalian cells by oxidant stress, is known to be part of a general inducible antioxidant defence pathway. The fact that prostaglandin synthesis is stimulated in muscle during contraction and in the heart in response to ischaemia raises the possibility that induction of haem oxygenase by PGA in myoblasts could be part of a protective mechanism in operation during stress and hypoxia.

INTRODUCTION

Prokaryotic and eukaryotic cells exhibit an essential, highly conserved and finely regulated cellular response to adverse environmental conditions, including heat shock, anoxia, exposure to transition heavy metals and amino acid analogues, by inducing the synthesis of a specific set of proteins referred to as heat shock proteins or stress proteins [1]. The activation of heat shock gene transcription is mediated by a transcription factor known as heat shock factor, which binds to heat shock elements composed of multiple adjacent inverted repeats of the pentamer nGAAn in the promoters of heat shock genes. The presence of multiple heat shock factors has now been described in several species, including humans [2,3]. Stress proteins can be divided into five families, depending on their molecular mass. In eukaryotic cells 70 kDa heat shock proteins (hsp70), which are encoded by a large multigene family, include the constitutively expressed hsc70, the major inducible hsp70, the inducible hsp72, the glucose-regulated grp78/BiP and the mitochondrial P75 (reviewed in ref. [4]). Although a nucleotide sequence comparison of hsp70-related genes from different species has revealed a high degree of evolutionary conservation (for example, *Escherichia coli* dnaK and human hsp70 are 50% identical at the amino acid level), different mechanisms of induction have been described in different organisms [2–4].

Abnormal expression of stress proteins has been described in a variety of pathophysiological states, which include fever, inflammation, viral infection, cancer, ischaemia and oxidant injury [5]. Stress proteins, however, are far more than just defensive molecules. They participate in essential metabolic processes, including protein folding and intracellular translocation, the control of cell-cycle progression, development and differentiation, and their synthesis can be induced by specific bioactive molecules, including haemin and prostaglandins [5–9].

Prostaglandins are synthesized almost universally in eukaryotic cells and function as microenvironmental hormones, participating in the regulation of a large variety of physiological and pathological processes, including muscle contraction [10], fever [11], cell proliferation and differentiation [12] and virus infection [13,14]. Prostaglandins of the A type (PGAs), which are derived from E-type prostaglandins by dehydration in plasma or aqueous solution [15], are known to inhibit cell proliferation by blocking cell-cycle progression in the G1 phase and to induce differentiation in several types of cells [14,15]. PGAs are also effective inhibitors of replication of RNA and DNA viruses in different types of mammalian cells, as well as in animal models [13–15]. Both the antiproliferative and antiviral activity have been related to the ability of PGA to induce hsp70 expression [7,16]. Recently induction of hsp70 has been associated also with a cytoprotective effect of PGA_1 after thermal injury [17].

Induction of hsp70 synthesis by PGA has been described in several monkey (MA104, 37RC, VERO), canine (MDCK), porcine and human cell lines, including K562 erythroleukaemia, HeLa cells, MT-2 leukaemia, human peripheral blood lymphocytes and primary cells derived from cord blood (reviewed in refs. [8] and [14]). In human K562 erythroleukaemic cells, induction of hsp70 by PGA_1 is mediated by cycloheximide-sensitive heat shock factor activation [9], and is generally accompanied by induction of synthesis of hsp90 and hsp110, whereas hsp60 and the constitutively expressed 70 kDa heat shock protein (hsc70) are not affected at the transcriptional level [7,9].

In the present report we provide evidence that, in murine myoblasts, PGA_1 increased the synthesis of hsc70 but was not able to induce hsp70 synthesis. Instead it dramatically induced the synthesis of a 32 kDa protein, which we have identified as haem oxygenase (HO).

EXPERIMENTAL

Cell culture and treatment with heat, PGA_1 and chemicals

C2C12 mouse myoblasts, kindly provided by Stefano Alemà

Abbreviations used: HO, haem oxygenase; hsp70, 70 kDa heat shock protein; hsc70, constitutive 70 kDa heat shock protein; PGA_1, prostaglandin A_1; GAPDH, glyceraldehyde phosphate dehydrogenase; BSO, D,L-buthionine-*S*,*R*-sulphoximine; DEM, diethylmaleate; DMEM, Dulbecco's modified Eagle's medium.

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(Institute of Cell Biology, CNR, Rome, Italy), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (Gibco), 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere.

For the heating procedure, dishes were submersed in a temperature-controlled water-bath (Grant Instruments, Cambridge, U.K.) at 45° ± 0.01 °C for 20 min ($t_{1/2}$ = 1.5 min). PGA_1 and other prostaglandins (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) were stored as 10 mg/ml ethanolic stock solution at −20 °C, and were diluted to the appropriate concentration in culture medium immediately before use. Control medium contained the same concentration of ethanol diluent, which did not affect cell viability or protein synthesis. Sodium *m*-arsenite (Sigma, St. Louis, MO, U.S.A.) in aqueous solution was used at a final concentration of 80 μM, unless otherwise specified.

Protein synthesis and PAGE

C2C12 myoblasts, inoculated 2 days before treatment in 35 mm plastic dishes, were treated by heat shock, or with PGA_1 or sodium arsenite and labelled in the same medium for 7 h with 12 μCi/ml L-[^{35}S]methionine or 15 μCi/ml L-[^{3}H]leucine. For the time-course experiments, at each time indicated the medium was replaced with methionine-free medium and 10 μCi/ml L-[^{35}S]methionine was added after 15 min. The radioactivity incorporated into trichloroacetate-insoluble material was determined as described previously [18], and L-[^{35}S]methionine- or L-[^{3}H]leucine-labelled samples containing the same amount of radioactivity were separated by SDS/PAGE in a vertical slab gel apparatus (3% stacking gel, 10% resolving gel unless otherwise specified) and subjected to autoradiography [18]. Gels containing L-[^{3}H]leucine were treated for fluorography with Amplify (Amersham International, Amersham, Bucks., U.K.). Densitometric analysis of autoradiograms was performed on a Bio-Rad model 620CCD videodensitometer (Bio-Rad Laboratories, Richmond, CA, U.S.A.), and protein was determined using Bio-Rad 1-D Analyst software. Synthesis of HO, hsc70 and hsp70 was expressed as percentage of total protein synthesis.

Immunoblot analysis

Equal amounts of protein for each sample were separated by SDS/PAGE and blotted to nitrocellulose, as described by Burnette [19]. After transfer, the filters were incubated with monoclonal anti-hsp70 antibodies (3A3 monoclonal antibodies kindly provided by R. Morimoto, Northwestern University, Evanston, IL, U.S.A.), which recognize both the 73 kDa constitutive protein, hsc, and the 72 kDa inducible protein, hsp, diluted 1:100 in TEN/Tween 20 buffer (0.05 M Tris/HCl, pH 7.4, 5 mM EDTA, 0.15 M NaCl, 0.05% Tween 20). The bound antibody was detected by horseradish peroxidase-linked sheep anti-mouse antibody (Amersham), as previously described [7].

Two-dimensional gel electrophoresis

C2C12 myoblasts treated for 5 h with PGA_1 or sodium arsenite were placed in prewarmed medium lacking methionine. L-[^{35}S]Methionine (25 μCi/ml) was added and cells were incubated for the following 2 h at 37 °C. Incorporation of L-[^{35}S]methionine was terminated by washing with ice-cold PBS. The cells were scraped off and lysed in sonication buffer [0.01 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 1% (w/v) Nonidet P40, 50 μg/ml RNAase A and protease inhibitors] by the method of O'Farrell [20]. Equal amounts of protein (20 μg) for each sample were run into isoelectric focusing tube gels (inner diameter 1.5 mm) composed of 9.2 M urea, 2% Nonidet P40, 4% acrylamide/bisacrylamide (from a 30% stock solution of 28.4% acrylamide and 1.6% bisacrylamide) and 2% Ampholine (1.5% pH range 5–7 and 0.5% pH range 3–10). After the run (300 V for 13 h and 800 V for 2 h), the isoelectric focusing gels were extruded from the tube, equilibrated for 15 min at room temperature in SDS sample buffer [10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% SDS, 0.0625 M Tris/HCl, pH 6.8, and 0.01% Bromophenol Blue] and run in the second dimension on 10% polyacrylamide slab gels. After electrophoresis, the gels were treated as described for one-dimensional gels. The isoelectric point of p32 was 6.4.

RNA extraction and Northern-blot analysis

Before harvesting, each plate was washed with PBS. The cells were scraped off and transferred to Eppendorf tubes and then lysed in 400 μl of lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.4, and 0.5% Nonidet P40). After centrifugation the nuclear pellet was discarded, 10 × proteinase K buffer (2 M $LiCl_2$, 100 mM Tris/HCl, pH 8, 100 mM EDTA and 5% SDS) (40 μl) and proteinase K (200 μg) were added to each supernatant, and the mixture was incubated at 45 °C for 2 h. After extraction with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and then chloroform/3-methylbutan-1-ol (24:1, v/v), RNA was precipitated with 2.5 vol. of ethanol, and quantified. Equal quantities of RNA (15 μg) were loaded into each slot of Mops/formaldehyde/1% agarose gels. After electrophoresis RNA was capillary-blotted on to nylon membranes (Amersham Hybond-N+). Membranes were baked, prehybridized and hybridized at 42.5 °C to ^{32}P-labelled probes prepared by using random-primed DNA synthesis of a 1 kb *Eco*RI fragment of cDNA human HO clone 2/10 [21] (a gift from Rex M. Tyrrell, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). After being stripped, filters were then rehybridized with ^{32}P-labelled probe derived from a cDNA fragment (1400 bp; *Pst*I) of the rat glyceraldehyde phosphate dehydrogenase (GAPDH) gene as a loading control, and then with ^{32}P-labelled hsp70 probe, pH2.3 plasmid [22], kindly provided by R. Morimoto. Radioactivity was quantified by using a Molecular Dynamics Phosphoimager analyser (MDP, Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Transcriptional run-on assay

In vitro run-on transcription reactions were performed in isolated C2C12 nuclei as described [23]. ^{32}P-labelled RNA was used for hybridization to nitrocellulose filters containing plasmids for the HO gene (clone 2/10) or the GAPDH gene as control. After hybridization, filters were visualized by autoradiography and the radioactivity was quantified by use of a Molecular Dynamics Phosphoimager analyser.

Measurement of GSH

After treatment with prostaglandins, control diluent or other agents, cell monolayers were rinsed with PBS, treated with trypsin, resuspended in cold PBS, counted and pelleted by centrifugation at 4 °C. The cell pellets were extracted with 5% metaphosphoric acid. After centrifugation at 4 °C, total intracellular GSH levels were measured at 25 °C using the colorimetric GSH-400 assay (Bioxytech S.A., Bonneuil/Marne, France). The inhibitor of γ-glutamylcysteine synthetase, D,L-buthionine-*S*,*R*-sulphoximine (BSO; Sigma), was used at a concentration of 50 μM, and diethylmaleate (DEM; Sigma) was

used at a concentration of 100 μM. Data are expressed as percentage of the total intracellular GSH in untreated controls and represent means ± S.D. of three independent experiments.

RESULTS

Effect of PGA_1 on C2C12 myoblast protein synthesis

C2C12 mouse myoblasts (1×10^6/dish) were either heat-shocked at 45 °C for 20 min or treated with PGA_1 (30 μM) or ethanol diluent. Immediately after treatment, cells were labelled with L-[^{35}S]methionine for the next 7 h. After determination of the radioactivity incorporated into acid-insoluble material, samples were subjected to SDS/PAGE and autoradiography. Figure 1(a) shows that, whereas heat shock induced the synthesis of hsp70 in C2C12 cells as expected, PGA_1 surprisingly had no effect on hsp70 synthesis, and only slightly increased hsc70 synthesis. PGA_1, however, strongly induced the synthesis of a 32 kDa protein (p32) in these cells. Hsp70 was identified in the same samples by immunoblot analysis using 3A3 monoclonal antibodies which recognize both the constitutive (hsc70) and the inducible (hsp70) form (Figure 1b). Immunoblot analysis confirmed that hsp70 was induced by heat shock but not by PGA_1 in these cells. In a separate experiment C2C12 cells were treated as described above and labelled with L-[^{3}H]leucine (7 h pulse) starting after the heat shock or after addition of PGA_1. Under these conditions the synthesis of a low-molecular-mass heat shock protein (hsp28), which is normally poorly labelled with L-[^{35}S]methionine [24], was detected in heat-shocked but not PGA_1-treated cells (Figure 1c). Synthesis of p32 was instead detected in the PGA_1-treated cells under these conditions also.

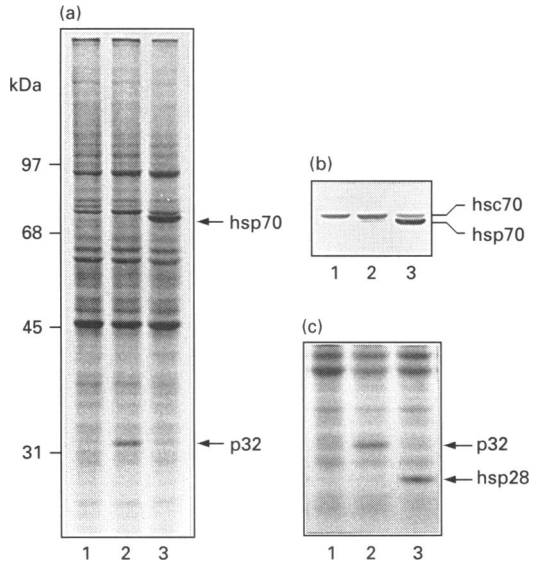

Figure 1 Effect of PGA_1 and heat-shock treatment on C2C12 myoblast protein synthesis

C2C12 cells treated with 30 μM PGA_1 (lane 2) or ethanol diluent (lane 1) at 37 °C, or heat-shocked at 45 °C for 20 min (lane 3) were labelled with L-[^{35}S]methionine (**a**) or L-[^{3}H]leucine (**c**) soon after treatment for the next 7 h. Samples containing equal amounts of radioactivity were subjected to SDS/PAGE and autoradiography. Samples containing equal amounts of protein were subjected to immunoblot analysis, using 3A3 monoclonal antibodies, which recognize both the constitutive (hsc70) and the inducible (hsp70) forms of the protein (**b**). PGA_1 did not induce hsp70 synthesis, but selectively induced the synthesis of a 32 kDa protein (p32). (**c**) Lower portion of the gel (12% resolving gel); hsp28 is the low-molecular-mass heat shock protein detected after L-[^{3}H]leucine labelling.

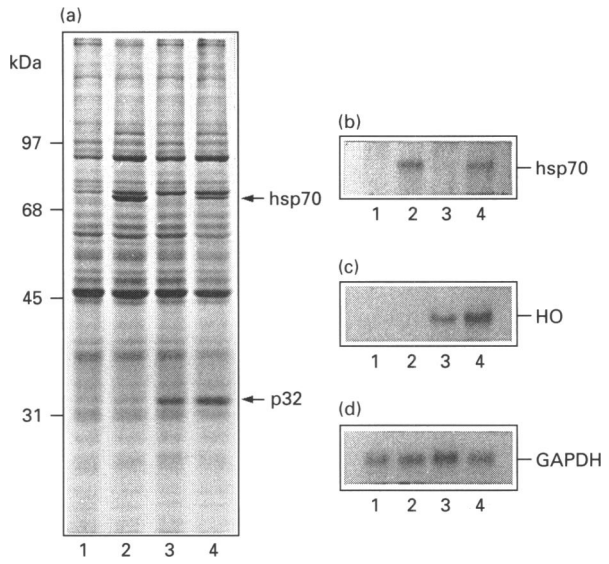

Figure 2 Identification of p32 as HO

C2C12 cells were treated with 30 μM PGA_1 (lane 3), 80 μM sodium arsenite (lane 4) or control diluent (lane 1) at 37 °C, or were heat-shocked at 45 °C for 20 min (lane 2). Cells were labelled with L-[^{35}S]methionine (7 h pulse) soon after heat shock or other treatments, and samples containing the same amount of radioactivity were subjected to SDS/PAGE and autoradiography (**a**). Cytoplasmic RNAs from unlabelled cells treated as in (**a**) were extracted 3 h after the end of heat-shock or beginning of PGA_1 or sodium arsenite treatment. Equal quantities of RNA (15 μg) were separated on a 1% agarose gel and subjected to Northern-blot analysis, using ^{32}P-labelled probes specific for the hsp70 gene (**b**), the HO gene (**c**) and the GAPDH gene, as a loading control (**d**).

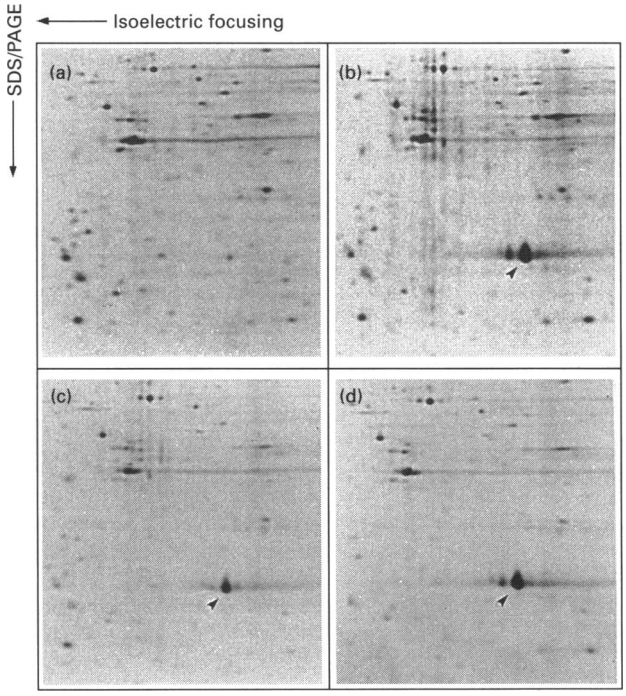

Figure 3 Comparison of PGA_1- and sodium arsenite-induced p32 by two-dimensional gel electrophoresis

C2C12 cells treated with 30 μM PGA_1, 80 μM sodium arsenite or ethanol (control) were labelled with L-[^{35}S]methionine (2 h pulse), starting 5 h after treatment. Samples containing equal amounts of protein (20 μg) were subjected to two-dimensional PAGE. (**a**) Control; (**b**) PGA_1; (**c**) sodium arsenite; (**d**) mixture of samples in (**b**) and (**c**). p32 (pI 6.4) is indicated by the arrowhead.

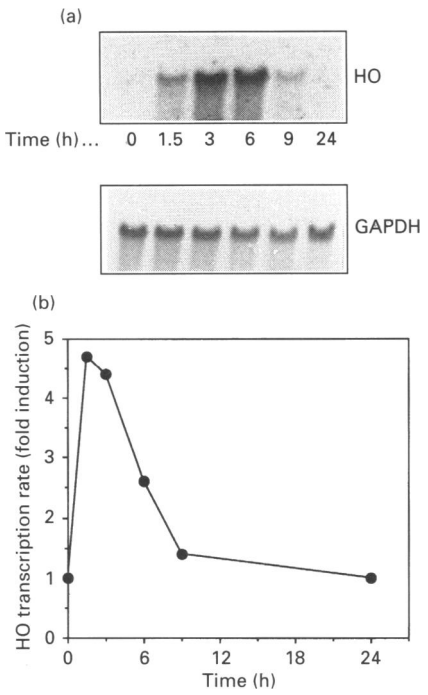

Figure 4 Kinetics of HO mRNA synthesis and accumulation after PGA_1 treatment

(**a**) C2C12 cells were treated with 30 μM PGA_1 or ethanol diluent. Cytoplasmic mRNA was extracted soon after (time 0) or at 1.5, 3, 6, 9 or 24 h after PGA_1 treatment, and subjected to Northern-blot analysis using the ^{32}P-labelled HO probe or the GAPDH probe as loading control. (**b**) In the same experiment the transcription rate of the HO gene and the GAPDH gene as control were analysed by run-on assay in isolated nuclei from cells treated with 30 μM PGA_1 or ethanol diluent. Radioactivity was quantified using a Molecular Dynamics Phosphoimager analyser. The values given for each time point were first corrected for the vector background and then normalized relative to the signal for GAPDH. Values are expressed as fold induction over control levels at time 0.

Identification of p32 as HO

It has been shown that oxidative stress induces the synthesis of a 32 kDa stress protein, which has now been identified as HO [21]. To investigate whether the PGA_1-induced p32 is HO, C2C12 cells were treated with PGA_1 (30 μM), sodium arsenite (80 μM), a known inducer of HO [21] or ethanol diluent at 37 °C, or were subjected to heat shock (45 °C, 20 min). Cells were labelled with L-[^{35}S]methionine soon after the various treatments for the next 7 h. SDS/PAGE analysis of radiolabelled samples showed that heat shock induced hsp70 synthesis but not p32 synthesis, PGA_1 induced p32 synthesis but not hsp70 synthesis as expected, and sodium arsenite induced synthesis of both (Figure 2a).

In the same experiment, in order to identify p32, cytoplasmic RNA was extracted from control, heat-shocked or PGA_1- or sodium arsenite-treated cells, 3 h after the end of heat shock or beginning of other treatment. Equal quantities of RNA (15 μg) were loaded on to agarose gels and, after electrophoresis, were blotted on to nylon membranes for hybridization with ^{32}P-labelled human HO cDNA, clone 2/10. After being stripped, filters were rehybridized with a ^{32}P-labelled probe derived from a cDNA fragment of the rat GAPDH gene as a loading control, and then with ^{32}P-labelled pH2.3 plasmid for identification of hsp70 RNA. Figure 2(b) shows the presence of hsp70 transcripts in heat-shocked and sodium arsenite-treated cells, but not in

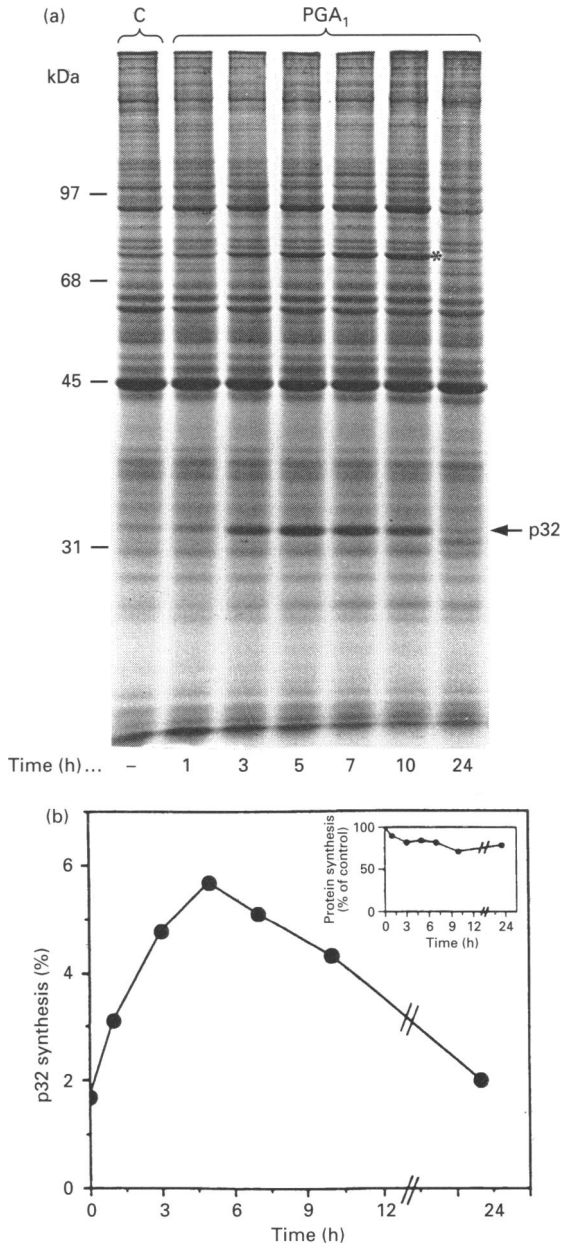

Figure 5 Kinetics of p32 synthesis after PGA_1 treatment in C2C12 cells

(**a**) C2C12 cells, treated with 30 μM PGA_1 or ethanol diluent (C), were labelled with L-[^{35}S]methionine (1 h pulse) at different times after treatment. Samples containing the same amount of radioactivity were subjected to SDS/PAGE and autoradiography. Apart from induction of p32 expression, an increase in the synthesis of hsc70 (indicated by the asterisk) can be detected from 5 to 10 h after PGA_1 treatment (2-fold increase at 10 h). (**b**) Quantitative determination of p32 synthesis by densitometric analysis of the gel in (**a**). The effect of PGA_1 on C2C12 total protein synthesis, determined in the same experiment, is shown in (**b**) (inset).

PGA_1-treated cells. HO RNA was detected in both sodium arsenite- and PGA_1-treated cells, but not in heat-shocked cells (Figure 2c).

HO induced by sodium arsenite or PGA_1 was also characterized by two-dimensional electrophoretic analysis of L-[^{35}S]methionine-labelled samples. C2C12 cells were treated with PGA_1 (30 μM), sodium arsenite (80 μM) or ethanol diluent and labelled 5 h after treatment for 2 h. Equal amounts of protein (20 μg) from each sample were run in isoelectric focusing tube

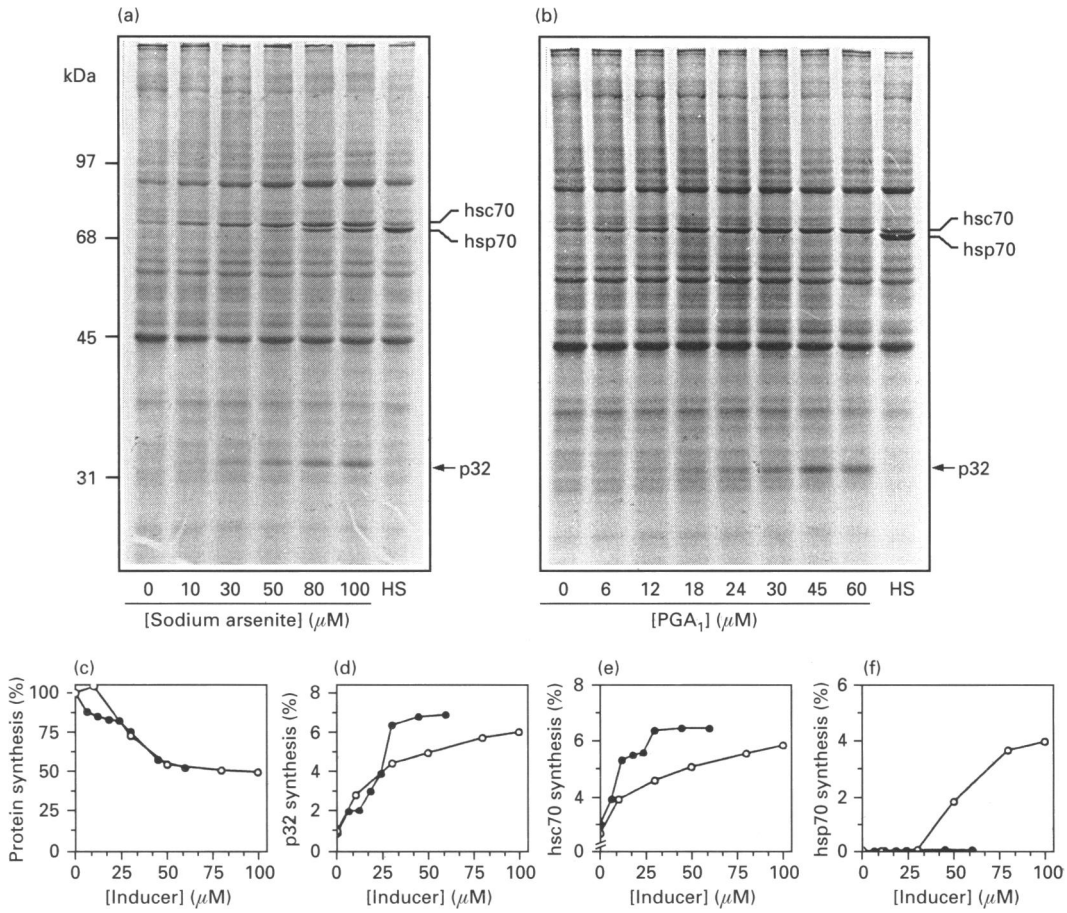

Figure 6 Dose-dependent effect of PGA_1 and sodium arsenite on synthesis of p32 and heat shock proteins

C2C12 cells were treated with increasing concentrations of sodium arsenite (**a**) or PGA_1 (**b**) at 37 °C, or heat shocked at 45 °C for 20 min, and labelled with L-[^{35}S]methionine (7 h pulse) soon after treatment. Samples containing the same amount of radioactivity were subjected to SDS/PAGE and autoradiography. HS, heat-shocked control cells. The effects of different concentrations of PGA_1 (●) or sodium arsenite (○) on total protein synthesis or synthesis of p32, hsc70 and hsp70 are shown in (**c**), (**d**), (**e**) and (**f**) respectively. Percentage of p32, hsc70 and hsp70 synthesis was determined by densitometric analysis of the gels in (**a**) and (**b**) as described in the Experimental section.

gels, as described in the Experimental section. Isoelectric focusing gels were then run in the second dimension on 10% polyacrylamide slab gels, and autoradiographed. Figure 3 shows that PGA_1-induced and sodium arsenite-induced p32 migrate identically.

Kinetics of HO induction by PGA_1

HO mRNA levels were determined in C2C12 cells treated with 30 μM PGA_1 or ethanol diluent. At different times after treatment, total cytoplasmic RNA was isolated and subjected to Northern-blot analysis, using the ^{32}P-labelled HO probe, clone 2/10, or the GAPDH probe as control. HO mRNA started to accumulate 1.5 h after PGA_1 treatment, reached maximal levels between 3 and 6 h, and went back to control levels 24 h after treatment (Figure 4a). To determine whether the accumulation of HO mRNA levels in the presence of PGA_1 was due to enhanced HO mRNA transcription or increased mRNA stability, the transcription rate of the HO gene was examined in isolated nuclei from untreated or PGA_1-treated cells by run-on analysis. PGA_1 was found to induce HO mRNA transcription (Figure 4b). HO mRNA transcription rate was maximal between 1.5 and 3 h after PGA_1 treatment, and went back to control level 24 h later.

We next investigated the kinetics of HO (p32) protein synthesis in PGA_1-treated cells. C2C12 monolayers were treated with PGA_1 (30 μM) or ethanol diluent and then labelled with L-[^{35}S]methionine (1 h pulse) at different times for the next 24 h. After labelling, cells were lysed, radioactivity incorporated into trichloroacetate-insoluble material was determined, and samples were subjected to SDS/PAGE and p32 determination. Figure 5(b) (inset) shows that, at this concentration, PGA_1 only slightly (10–20%) inhibited protein synthesis in these cells. Synthesis of p32 started at 3 h after PGA_1 treatment, reached a maximum between 5 and 7 h, and returned to control levels by 24 h. The level of the constitutive (hsc70) protein (but not hsp70) appeared to be increased in PGA_1-treated cells between 5 and 10 h after treatment.

Dose–response effect of PGA_1 and sodium arsenite on stress-protein synthesis in C2C12 cells

To investigate whether PGA_1 could induce the synthesis of hsp70 or other stress proteins at higher concentrations, and to compare the effect of PGA_1 on protein synthesis with the effect of sodium arsenite, C2C12 cells were treated with different concentrations of both drugs, or were subjected to heat shock at 45 °C for 20 min as a control. Cells were labelled with L-[^{35}S]methionine

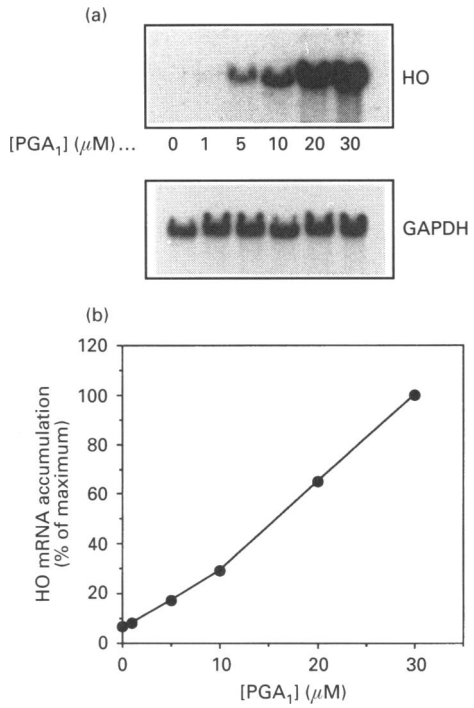

Figure 7 Dose-dependent increase in HO mRNA levels in C2C12 cells treated with PGA_1

(**a**) C2C12 cells were treated with increasing concentrations of PGA_1 in DMEM supplemented with antibiotics and glutamine, in the absence of fetal calf serum. Cytoplasmic mRNA was extracted 5 h after treatment and subjected to Northern-blot analysis using the ^{32}P-labelled HO probe or the GAPDH probe as control. (**b**) Radioactivity in samples in (**a**) was quantified using a Molecular Dynamics Phosphoimager analyser. The values are expressed as percentage of maximum level.

soon after treatment for the next 7 h, and the effect on general protein synthesis and synthesis of p32, hsc70 and hsp70 was determined. Figure 6 shows that p32 synthesis increased dose-dependently in PGA_1-treated cells, up to a concentration of 30–45 μM. Higher concentrations of PGA_1 did not increase p32 synthesis further. Induction of p32 was obtained at doses that only slightly inhibited cell protein synthesis (Figure 6c). PGA_1 also increased dose-dependently the synthesis of the constitutive protein, hsc70 (Figure 6e), but had no effect, even at high doses, on hsp70 synthesis (Figure 6f). Sodium arsenite was found to increase both p32 and hsp70 synthesis, as well as that of hsc70, in these cells.

In a separate experiment, levels of HO mRNA were determined by Northern-blot analysis in C2C12 cells treated with different concentrations of PGA_1 in DMEM in the absence of fetal calf serum, to avoid PGA_1 sequestering by serum albumin. As shown in Figure 7, a dose-dependent increase in HO mRNA accumulation was found 5 h after PGA_1 treatment. Under these conditions, concentrations of PGA_1 as low as 1–5 μM were effective at increasing HO mRNA levels.

Effect of PGA_1 and sodium arsenite on intracellular GSH levels in C2C12 cells

It has been suggested that a reduction in intracellular GSH concentration could be responsible for enhanced HO expression [25]. As PGAs have been shown to conjugate with GSH in a reaction catalysed by homogeneous glutathione *S*-transferase in several types of mammalian cells [15,26], we examined the possibility that HO induction by PGA_1 could be due to a reduction in GSH levels in C2C12 cells.

C2C12 cells were treated with PGA_1 at concentrations ranging from 10 to 90 μM. Each concentration was tested in duplicate samples, and duplicate controls containing identical amounts of

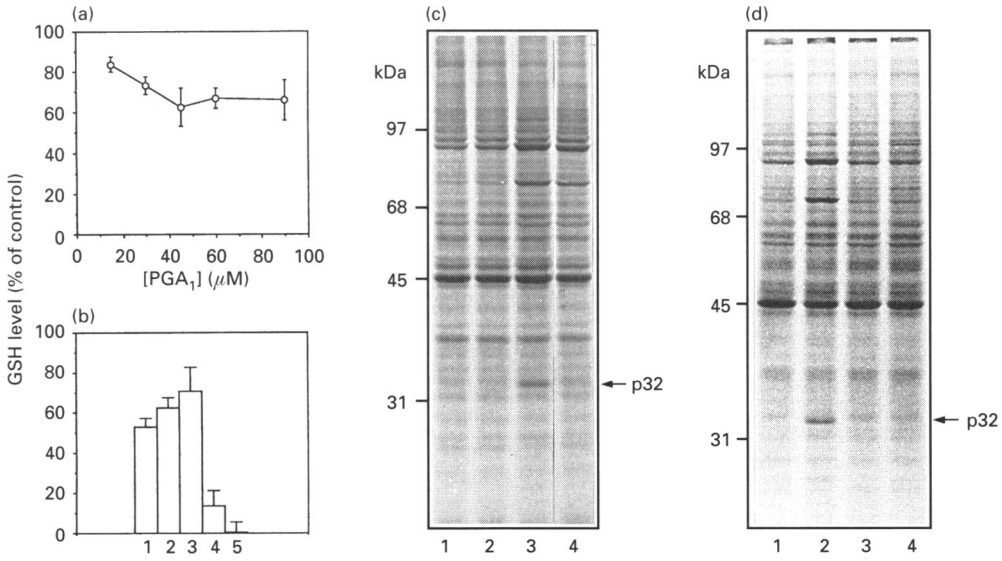

Figure 8 Effect of PGA_1 and other agents on intracellular GSH levels and HO induction

(**a**) C2C12 cells were treated with PGA_1 at different concentrations. After 3 h at 37 °C, the intracellular GSH content was determined, as described in the text. (**b**) C2C12 cells were treated with 80 μM sodium arsenite (1), 45 μM PGA_1 (2), 50 μM BSO (3), 45 μM PGA_1 + 50 μM BSO (4) or 100 μM DEM (5) as a control, and intracellular GSH levels were determined 3 h after the beginning of treatment. (**c**) C2C12 cells kept for 18 h in the presence of 50 μM BSO or control diluent were treated with PGA_1 (15 μM) or ethanol diluent, and labelled with L-[^{35}S]methionine for the next 7 h. Samples containing equal amounts of radioactivity were subjected to SDS/PAGE and autoradiography. Lane 1, control; lane 2, BSO; lane 3, BSO + PGA_1; lane 4, PGA_1. (**d**) C2C12 cells were treated with 45 μM PGA_1 (lane 2), PGE_2 (lane 3), $PGF_{2\alpha}$ (lane 4) or ethanol diluent (lane 1) and labelled with L-[^{35}S]methionine 5 h after treatment. Samples containing equal amounts of radioactivity were subjected to SDS/PAGE and autoradiography. In a parallel experiment intracellular GSH levels were determined 3 h after the beginning of treatment. PGA_1 was found to induce HO (indicated by arrow), and to decrease intracellular GSH levels (62.6 ± 4.9% of control), whereas PGE_2 and $PGF_{2\alpha}$ did not induce HO synthesis and did not significantly alter GSH levels (PGE_2 = 84.6 ± 10.1 and $PGF_{2\alpha}$ = 86.5 ± 6.9% of control).

ethanol diluent were tested for each concentration. After 3 h at 37 °C, a time at which HO mRNA was already abundantly expressed in PGA_1-treated cells, cells were washed with PBS, treated with trypsin and analysed for intracellular GSH content. PGA_1 treatment caused a dose-dependent depletion of intracellular GSH levels which was maximal (approx. 40%) at a concentration of 45 μM (Figure 8a). Higher concentrations of PGA_1 did not decrease GSH levels further. At a concentration of 45 μM, PGA_1 also caused maximal induction of HO synthesis (Figures 6b and 6d). C2C12 cells were then treated with 45 μM PGA_1, and intracellular GSH levels were determined at different times after PGA_1 addition. A decrease in intracellular GSH levels of approx. 26% was detected as early as 15 min and the maximum decrease (about 40%) was detected 3 h after PGA_1 addition, which was therefore selected as the time for GSH measurement in the following experiments. The decrease in intracellular GSH levels after treatment with 45 μM PGA_1 was comparable with that obtained after a 3 h treatment with 80 μM sodium arsenite (Figure 8b). The inhibitor of γ-glutamylcysteine synthetase, BSO, at a concentration of 50 μM caused a decrease in GSH levels of approx. 29% after 3 h, and simultaneous treatment with 50 μM BSO and 45 μM PGA_1 resulted in a GSH depletion of more than 85% (Figure 8b) and an increase in HO mRNA levels (results not shown). BSO (50 or 100 μM) was unable to induce HO synthesis in C2C12 cells even after 24 h of treatment (Figure 8c). However, BSO pretreatment (18 h) resulted in an approx. 2-fold increase in HO synthesis in cells treated with 15 μM PGA_1 compared with cells not treated with BSO (Figure 8c). BSO also increased HO synthesis after treatment with PGA_1 at higher concentrations. On the other hand, when C2C12 cells were treated with *N*-acetylcysteine (30 mM) for 2 h before PGA_1 treatment, PGA_1 was unable to induce HO synthesis (results not shown). DEM, which forms thioether conjugates with GSH and is a potent inducer of HO expression in different types of cell [27], at a concentration of 100 μM was found to cause a dramatic decrease in intracellular GSH levels (Figure 8b) and to induce HO protein synthesis in C2C12 cells (results not shown).

Finally the effect of prostaglandins that do not possess a cyclopentenone structure on HO expression and intracellular GSH concentration was investigated. C2C12 cells were treated with 45 μM PGA_1, PGE_2, $PGF_{2\alpha}$ or ethanol diluent, and labelled with L-[^{35}S]methionine (10 μCi/10^6 cells; 1 h pulse) 5 h after treatment. In a parallel experiment, intracellular GSH levels were determined 3 h after the beginning of treatment. In contrast with PGA_1, PGE_2 and $PGF_{2\alpha}$ did not induce HO expression (Figure 8d). Whereas PGA_1 caused the expected reduction in GSH levels, no significant alteration in intracellular GSH levels was found after treatment with PGE_2 and $PGF_{2\alpha}$.

Effect of PGA_1 on HO induction in different types of murine cells

An equal number of C2C12 myoblasts, L929 fibroblasts, N2A neuroblastoma and B16 melanoma cells were treated with PGA_1 (30 μM) and labelled with L-[^{35}S]methionine for the next 7 h. After cell lysis, the radioactivity incorporated into acid-insoluble material was determined and samples were subjected to SDS/PAGE. Protein synthesis was affected differently by 30 μM PGA_1 in different types of cell: it was not significantly altered in C2C12 cells, but was reduced by more than 60% in L929 cells. PGA_1 was found to induce HO only in C2C12 myoblasts, indicating a selective effect of PGA_1 depending on the cell type (results not shown).

DISCUSSION

HO plays a key role in physiological haem catabolism in mammals, catalysing the oxidative cleavage of haem at the α-methene bridge, with the formation of biliverdin [28]. The enzyme biliverdin reductase then converts biliverdin into bilirubin [29]. Under physiological conditions, HO activity is highest in the spleen, where senescent erythrocytes are sequestered and destroyed [30]. However, because of the essential role of haem in cell metabolism, the HO gene is ubiquitously expressed in all tissues examined, as well as in cultured cells [31]. HO is now thought to be the low-molecular-mass protein induced in mammalian cells by oxidant stress, and its synthesis is considered to be part of a general inducible antioxidant defence pathway [32]. An HO-dependent increase in ferritin after oxidant stress has also been recently reported [33], suggesting that elevated levels of newly synthesized ferritin may confer increased resistance to oxidative stress by sequestering intracellular free iron, and decreasing iron-catalysed free-radical reactions. Moreover, bilirubin has been recently shown to be an efficient radical scavenger, and its role as a physiological antioxidant in plasma has been suggested [34].

Recently, HO has been shown to be induced *in vivo* in the heart of rats subjected to hypoxia [35], and a protective antioxidant role for this enzyme was shown in a model of rhabdomyolysis *in vivo* [36].

HO activity is normally induced by its substrate haem or haem analogues, and by a variety of chemical agents and physical types of stress [31,32]. The agents that induce HO have been divided into two major groups: oxidants or compounds that can generate active intermediates (u.v. A radiation, menadione, phorbol 12-myristate 13-acetate, H_2O_2) and agents that interact with GSH or modify cellular GSH levels (BSO, iodoacetamide, $CdCl_2$, diamide, sodium arsenite) [32]. Induction of HO by interleukin 6 has been recently shown in human hepatoma cells [37], and induction of a 30 kDa stress protein after treatment with H_2O_2, $CdCl_2$ and cyclosporin has been shown in fetal mouse myocytes [38].

The results described in the present report show that PGA_1 is a potent inducer of HO expression in myoblastic cells. HO synthesis starts 3 h after PGA_1 addition and continues for at least 10 h, with a maximum peak between 5 and 7 h after treatment. As shown by run-on analysis, PGA_1 acts at the transcriptional level by inducing HO mRNA synthesis. Induction is dose-dependent and levels of HO comparable with that achieved after treatment with the classical inducer sodium arsenite can be obtained at non-toxic concentrations, which do not inhibit cell protein synthesis.

The mechanism of HO induction is not completely understood. Although hyperthermia normally does not induce HO in mammalian cells, the rat HO gene contains a functional heat shock element, and exposure of rat glioma cells, myoblasts or hepatocytes to elevated temperatures causes a rapid increase in HO synthesis, indicating that this enzyme is a heat shock protein in rat [39,40]. In human cells, induction of HO expression associated with hsp70 synthesis has been reported in hepatoma cell lines after heat shock treatment [41], and in macrophages after erythrophagocytosis [42]. Moreover, several inducers of HO (e.g. sodium arsenite) also induce hsp70 synthesis [43].

In murine C2C12 myoblasts, sodium arsenite was found to induce both HO and hsp70 expression, but synthesis of HO could be obtained at concentrations (< 50 μM) that did not induce hsp70 synthesis. Induction of HO expression by PGA_1 was independent of hsp70 synthesis, which was not induced even at the highest concentration tested in these cells. Moreover a heat shock at 45 °C for 20 min induced high levels of hsp70 synthesis without altering HO expression in these cells. These results are consistent with the observation of Alam et al. [44] that incubation

of mouse hepatoma cells at 42 °C did not significantly induce HO expression, and reinforce the hypothesis that mouse HO is not a heat shock protein.

It has been recently suggested that the signal for HO induction may be related to modified or reduced levels of GSH, and that depletion of this ubiquitous non-protein thiol may lead to enhanced HO gene expression, as a result of either accumulation of active oxygen species or the direct influence of GSH on a critical target involved in signal transduction [25,45].

Prostaglandins of the A type possess an α,β-unsaturated carbonyl group in the cyclopentane ring, which renders this portion of the molecule able to form Michael's adducts with cellular nucleophilics, to covalently bind to cysteine residues of proteins via a thioether bond [15,46] and to conjugate with GSH in a reaction catalysed by homogeneous glutathione S-transferase in human, rat and avian cells [26]. This suggested that induction of HO by PGA_1 could be due to a reduction in GSH levels caused by conjugation of GSH with PGA_1 itself. In fact, PGA_1 treatment was found to cause a dose-dependent reduction in intracellular GSH levels in C2C12 cells. The maximum decrease in intracellular GSH levels (about 40%) was detected 3 h after treatment at a concentration of 45 μM, which also caused maximal induction of HO synthesis. The reduction in intracellular GSH levels after treatment with 45 μM PGA_1 was comparable with that obtained after 3 h treatment with sodium arsenite at a concentration of 80 μM. The possibility that the decrease in intracellular GSH level after prostaglandin treatment is due to conjugation of GSH to PGA_1 is supported by the finding that PGA_1–GSH conjugates are rapidly exported by a vanadate-sensitive ATP-dependent GSH S-conjugate export pump [47] from C2C12 cells (P. I. Homem de Bittencourt and M. G. Santoro, unpublished work). Prostaglandins of the E and F type, which do not possess an α,β-unsaturated carbonyl group in the cyclopentane ring and do not conjugate with GSH [15], did not alter intracellular GSH levels and were not able to induce HO synthesis in these cells. The cyclopentenone prostaglandins PGJ_2 and Δ^{12}-PGJ_2, which also conjugate with intracellular GSH [48], as well as DEM and arsenite were reported to induce the synthesis of a 32 kDa protein and stimulate HO activity in porcine aortic endothelial cells [49,50].

A 3 h treatment with BSO, an inhibitor of γ-glutamylcysteine synthetase, caused a decrease in GSH levels of less than 30%, whereas the simultaneous treatment with 45 μM PGA_1 and BSO resulted in a decrease of approx. 90%. Apart from the ability to conjugate with GSH, PGA has been shown to induce γ-glutamylcysteine synthetase in murine cells [51]. The synergistic effect of PGA_1 and BSO could therefore be due to the fact that BSO-treated cells could not compensate for the intracellular GSH loss by increased GSH synthesis after PGA_1 treatment.

Treatment with BSO potentiated PGA_1-induced HO synthesis, whereas treatment with *N*-acetylcysteine, which raises the intracellular concentration of cysteine and hence of GSH, had the opposite effect, preventing HO induction by PGA_1. These results all indicate that HO induction by PGA_1 is associated with decreased levels of GSH. However, in contrast with results from other cell types [52], GSH depletion obtained after treatment with BSO alone, even prolonged (24 h) treatment, was not a sufficient condition for induction of HO in C2C12 cells. The inability of BSO to induce HO synthesis indicates that GSH depletion is not by itself sufficient to induce HO and that GSH conjugation is necessary for the induction signal, as previously proposed in a different model [45].

The prostaglandin precursor, arachidonic acid, has been shown to cause oxidative perturbation in cultured cells [53]. The possibility that PGA_1 may simply function as a general oxidant cannot be excluded. However, the fact that, even at high concentrations, PGA_1 does not induce HO in four different murine cell lines or in other cell types of different animal origin, in which it induces other stress proteins (reviewed in refs. [8] and [14]), indicates a selective effect of PGA_1 on specific cell types and argues against this possibility.

The finding that PGA_1 induces HO in myoblasts is particularly interesting in view of the fact that synthesis of prostaglandins of the E and A type is stimulated in muscle during contraction [10], and in dog and rabbit heart in response to ischaemia [54,55]. Induction of HO by PGA_1 in myoblastic cells could therefore be part of a protective mechanism during stress or hypoxia. It should be pointed out that, in our model, concentrations of PGA_1 higher than 1 μM were necessary for HO induction. Whether the levels of cyclopentenone prostaglandins could achieve a sufficiently high local concentration to induce HO in pathological conditions that cause an increase in arachidonic acid metabolism, such as hyperthermia [56], ischaemia [57] or exposure to u.v. irradiation [58], remains to be established.

The second observation emerging from the current study is that PGA_1, even at the highest concentration tested, was unable to mimic heat shock and induce hsp70 synthesis in C2C12 cells, although it increased hsc70 synthesis. Lack of hsp70 induction by PGA_1 was found in several other murine cell lines, including L929 fibroblasts, N2A neuroblastoma, B16 melanoma and FLC erythroleukaemic cells (A. Rossi and M. G. Santoro, unpublished work), indicating that murine cells behave differently from cells of other mammalian species in their response to PGA_1. The lack of hsp70 induction by PGA_1 in C2C12 cells could be related to the peculiar control mechanism of hsp70 gene expression by the murine heat shock transcriptional factors mHSF1 and mHSF2, compared with those from other vertebrate species [2,3]. In this case, the ability of PGA_1 to stimulate hsc70 synthesis without inducing hsp70 could be a useful tool in the study of activation and function of heat shock transcriptional factors in mouse.

The PGA_1-induced increase in hsc70 expression is also an intriguing finding. Developmentally regulated expression of heat shock proteins in unstressed murine cells has been reported during mouse embryogenesis and differentiation [59,60]. As PGAs induce differentiation in several experimental models (reviewed in refs. [12] and [15]), and PGE, the precursor of PGA, positively regulates myoblast differentiation [61], the possibility that the increase in hsc70 synthesis is correlated with a differentiating process is worth investigating.

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