Inhibition of cytokine-induced inducible nitric oxide synthase expression by glucagon and cAMP in cultured hepatocytes

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Addition of lipopolysaccharide plus interferon γ , tumour necrosis factor α and interleukin 1β to cultured hepatocytes resulted in the induction of inducible nitric oxide synthase (iNOS) activity as measured by $\text{NO}_3^- + \text{NO}_2^-$ formation, the conversion of L-[U-¹⁴C]arginine into citrulline and Western blotting of the iNOS protein. The inclusion of 1 μM glucagon during the induction period significantly decreased the effect of the cytokines on iNOS activity, the major effect being at the level of the total amount of protein, rather than alterations in substrate supply or covalent modification of the existing protein. In contrast, 1 μM insulin

was without effect. The effect of glucagon was mediated via cAMP and could be mimicked by the presence of either dibutyryl cAMP or forskolin to activate adenylate cyclase directly. It was rapid in onset and long-lived, a 30 min pre-treatment period protecting the cells from the induction of NO synthesis over the next 21 h in the presence of cytokines. Addition of glucagon at any time point up to 9 h after treatment of the cells with lipopolysaccharide plus the cytokines resulted in a significant inhibition of iNOS activity, glucagon being most potent when added during the first 3 h.

INTRODUCTION

It has been clearly demonstrated that the induction of inducible nitric oxide synthase (iNOS) activity within the liver is a major consequence of endotoxic shock and that this effect can be mimicked *in vitro* by the treatment of hepatocytes with lipopolysaccharide (LPS) plus a combination of cytokines including tumour necrosis factor α (TNF- α), interleukin 1β (IL- 1β) and interferon γ (IFN- γ) [1–4]. A major feature of both sepsis and endotoxin administration to animals is hyperglucagonaemia and a decrease in the plasma insulin-to-glucagon ratio [5–9]. In preliminary studies we have demonstrated that the presence of glucagon can antagonize the induction of iNOS by cytokines in cultured hepatocytes, as determined by a lowering of $\text{NO}_3^- + \text{NO}_2^-$ formation, and attenuate the inhibition of hepatic glucose output by the cytokines [10]. The interaction of hormones signalling via cAMP and agents that elevate cAMP levels with the expression of iNOS is complex and seems to be tissue-dependent. In vascular smooth-muscle cells, cAMP has been reported to synergize with cytokines such as IL- 1β and IFN- γ to increase the induction of iNOS [11–14], whereas increasing cAMP levels in the absence of cytokines results in either a small increase [12, 14] or no change in expression of the enzyme [13]. In rat renal mesangial cells cAMP results in a similar small elevation of iNOS synthesis but synergizes strongly with TNF- α and IL- 1β to enhance iNOS expression [15–17]. cAMP has also been reported to enhance iNOS expression and activity in response to IL- 1β in cardiac myocytes [18], whereas in cultured rat astrocytes noradrenaline and dibutyryl cAMP were shown to inhibit LPS-induced iNOS expression [19].

The aim of this study was to investigate further the effects of glucagon and cAMP on iNOS expression in cultured hepatocytes to determine whether the elevated glucagon levels observed during endotoxic shock might act together with elevated gluco-

corticoid levels to minimize iNOS expression and decrease the putative effects of NO on liver metabolism.

EXPERIMENTAL

Materials

Williams' Medium E, LPS (a trichloroacetic acid extract from *Salmonella typhimurium*), antibiotics, *N*⁶,2'-*O*-dibutyryl adenosine 3',5'-cyclic monophosphate (dibutyryl cAMP), forskolin, horseradish peroxidase-conjugated anti-(rabbit IgG), L-*N*^ω-monomethyl-L-arginine (L-NMMA) and collagenase were obtained from Sigma Chemical Co. (Poole, Dorset, UK). The newborn calf serum and recombinant rat IFN- γ were obtained from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). Recombinant human TNF- α was obtained from Calbiochem-Novabiochem (Nottingham, Notts., U.K.). Recombinant human IL- 1β was a gift from Dr. K. Ray (Glaxo-Wellcome, Stevenage, Herts., U.K.). The rabbit polyclonal iNOS antibody was obtained from Transduction Laboratories (Affinity Research Products, Exeter, Devon, U.K.). L-[U-¹⁴C]Arginine was obtained from ICN Biomedicals (Thame, Oxon., U.K.). L-[ureido-¹⁴C]Citrulline was obtained from NEN-Du Pont (Stevenage, Herts., U.K.). AG 50W-X8 resin was obtained from Bio-Rad (Hemel Hempstead, Herts., U.K.). All other reagents were from Sigma or BDH Laboratory Supplies (Poole, Dorset, U.K.).

Preparation and culture of hepatocytes

Male Sprague-Dawley rats (180–220 g body weight) were starved for 18 h and hepatocytes were prepared as described previously [20]. The hepatocytes were resuspended to a final concentration of 4.2×10^5 cells/ml in Williams' Medium E supplemented with 25 mM Hepes (pH 7.4)/2 mM glutamine/5% (v/v) heat-inactivated neonatal calf serum/ 10^5 i.u./l penicillin/100 mg/l

Abbreviations used: IFN- γ , interferon γ ; IL- 1β , interleukin 1β ; iNOS, inducible nitric oxide synthase; L-NMMA, L-*N*^ω-monomethyl-L-arginine; LPS, lipopolysaccharide; TNF- α , tumour necrosis factor α .

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streptomycin/1 μ M insulin/100 nM dexamethasone. Cells (5 ml) were plated on a 60 mm Primaria dish (Falcon; Becton Dickinson, Oxford, U.K.) to give a cell density of 7.5×10^4 cells/cm². After 2 h the medium was replaced with 2.5 ml of fresh medium without the serum and containing the hormones as indicated. The cells were treated for a further 21 h in the presence or absence of cytokines plus LPS and any other additions as stated in the text. The cytokines and LPS were added to the following final concentrations: IFN- γ , 100 i.u./ml; TNF- α , 500 i.u./ml; IL-1 β , 100 i.u./ml; LPS, 10 μ g/ml. This combination of cytokines was based on those used in previous studies to demonstrate maximal induction of iNOS in cultured hepatocytes and alterations in protein and carbohydrate metabolism [1,4,21]. Where appropriate, glucagon and dibutyryl cAMP were added to a final concentration of 1 μ M and forskolin was added to a final concentration of 100 μ M. At the end of the cytokine treatment period, the medium was removed and retained for the determination of NO₂⁻ + NO₃⁻ and the cells were rinsed in ice-cold 0.9% saline to remove any arginine carried over from the medium, followed by 1.0 ml of ice-cold extraction buffer containing 320 mM sucrose, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10 μ g/ml leupeptin, 0.1 mM PMSF, 10 μ g/ml soybean trypsin inhibitor and 10 μ g/ml aprotinin. The cells were scraped off the plate, immediately sonicated and frozen in liquid nitrogen for the subsequent determination of iNOS activity or for use in Western blotting for iNOS. iNOS activity in the cells was estimated either by the production of NO₂⁻ + NO₃⁻ in Williams' Medium E during the 24 h treatment period with cytokines or by the conversion of L-[U-¹⁴C]arginine to citrulline. NO₂⁻ + NO₃⁻ were assayed with the Griess reagent after conversion of the NO₃⁻ to NO₂⁻ with formate-nitrate reductase and standards prepared in Williams' Medium E to account for any non-cellular NO₃⁻ or NO₂⁻ production [22]. Williams' Medium E incubated in the absence of cells showed no detectable NO₃⁻ or NO₂⁻ production. The conversion of L-[U-¹⁴C]arginine to citrulline followed by separation by ion-exchange chromatography on Bio-Rad AG50W-X8 was as described by Salter et al. [23]. The incubation medium contained 50 mM Tris/HCl, pH 7.4, 2 mM CaCl₂, 0.2 mM NADPH, 50 mM valine, 1 mM citrulline, 5 mM GSH, 10 μ M tetrahydrobiopterin and 20 μ M L-[U-¹⁴C]arginine (specific radioactivity 0.7 μ Ci/ μ mol) (final concentrations). The incubation was performed for 10 min at 37 °C in the presence and the absence of 1 mM L-NMMA to correct for any residual arginase activity. Experiments performed with purified arginase in the presence and the absence of valine indicated that at the valine and arginine concentrations used, arginase activity was inhibited by $91.4 \pm 5.1\%$ (mean $\pm$ S.E.M.; $n = 5$). Similarly, experiments performed with cell extracts in the presence of 1 mM L-NMMA and in the presence or absence of valine indicated that the conversion of [U-¹⁴C]arginine was also inhibited by $90.9 \pm 1.7\%$ ($n = 4$), suggesting that the blank value obtained in the presence of L-NMMA represented arginase contamination in the assay. Citrulline recovery in the assay was determined by replacing the [U-¹⁴C]arginine with L-[ureido-¹⁴C]citrulline (0.1 mCi/ml) in the absence of the cell extract. Typical citrulline recoveries were $91.3 \pm 1.2\%$ ($n = 5$). To determine whether the labelled citrulline was being metabolized in these assays, leading to an underestimate of iNOS activity, the experiments were also performed in the presence of the cell extracts. The results indicated that only negligible citrulline metabolism occurred, the disappearance of citrulline being $1.4 \pm 0.5\%$ ($n = 4$) of the total citrulline added over the 10 min incubation period of the iNOS assay. Measurements of iNOS activity in the absence of Ca²⁺ and the presence of 1 mM EDTA showed no significant difference in activity (results not shown),

indicating that there was no significant Ca²⁺-dependent constitutive NOS activity in these cell extracts. For the detection of the iNOS by Western blotting, the protein samples were diluted 1:1 with sample buffer containing 5 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.2 M dithiothreitol and 0.02% Bromophenol Blue, boiled for 5 min and placed immediately on ice. The samples (10 μ g of protein) were separated by SDS/PAGE [8% (w/v) gel] and transferred to PVDF membrane. The membrane was stained in Ponceau S' and scanned to ensure even loading of the gel. It was then blocked for 1 h in 5% (w/v) non-fat milk powder and then the blot was incubated with anti-iNOS (diluted 1:2000). After the membrane had been washed, it was incubated with horseradish peroxidase-conjugated anti-(rabbit IgG) and the iNOS was detected by enhanced chemiluminescence. In the dose-response measurements, the cells were plated in six-well plates at the same density and after removal of the medium were immediately solubilized in boiling buffer containing 1% (w/v) SDS and 10 mM Tris/HCl, pH 7.0. The solubilized cells were then diluted 1:1 in Western blot sample buffer, boiled and blotted as described above. Protein was measured by the method of Lowry et al. [24] in all experiments except the dose-response measurements, where the bicinchoninic acid method was used [25].

The results are expressed as means $\pm$ S.E.M. with the number of individual cell preparations indicated. The level of significance of differences between the means was calculated by using a paired *t* test.

RESULTS AND DISCUSSION

Effect of insulin and glucagon on NO formation, iNOS activity and protein levels

Table 1 shows the effect of treatment of the cells with LPS plus a combination of IFN- γ , TNF- α and IL-1 β . In agreement with previous studies [1–4], this combination resulted in a significant increase in the induction of iNOS activity as measured by both NO₃⁻ + NO₂⁻ production and the incorporation of L-[U-¹⁴C]arginine into citrulline. The activity of the iNOS measured in

Table 1 Effect of glucagon on the induction of iNOS in the presence of insulin and dexamethasone

Cells were cultured as described in the Experimental section with 1 μ M insulin, 100 nM dexamethasone or vehicle in the presence or absence of IFN- γ (100 i.u./ml), TNF- α (500 i.u./ml), IL-1 β (100 i.u./ml) and LPS (10 μ g/ml). Where appropriate, glucagon was added simultaneously with the cytokines to a final concentration of 1 μ M. Samples were removed from the medium after 21 h for the determination of NO₃⁻ + NO₂⁻ production and the cells were processed for the measurement of iNOS activity. The results are expressed as means $\pm$ S.E.M. for five or six different cell preparations. All the cytokine-treated cells in the absence of glucagon were significantly different from their appropriate controls at the level of at least $P < 0.01$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for significance of difference from the appropriate treatment in the absence of glucagon.

Treatment	Glucagon	NO ₃ ⁻ + NO ₂ ⁻ production (nmol/mg of protein)		iNOS activity (pmol/min per mg of protein)	
		— Cytokines	+ Cytokines	— Cytokines	+ Cytokines
Control	—	25.4 $\pm$ 11.3	335.2 $\pm$ 13.7	0.8 $\pm$ 0.6	54.7 $\pm$ 17.0
	+	3.7 $\pm$ 1.9	65.7 $\pm$ 24.1***	0.3 $\pm$ 0.2	16.0 $\pm$ 6.3*
Insulin	—	25.9 $\pm$ 11.9	326.2 $\pm$ 32.1	2.0 $\pm$ 1.2	54.1 $\pm$ 11.3
	+	8.5 $\pm$ 4.4	57.1 $\pm$ 17.3***	2.2 $\pm$ 1.0	11.5 $\pm$ 6.2*
Dexamethasone	—	15.4 $\pm$ 4.6	225.9 $\pm$ 37.6*	3.3 $\pm$ 1.6	37.9 $\pm$ 4.7
	+	5.3 $\pm$ 3.7	40.8 $\pm$ 20.3**	3.6 $\pm$ 1.4	7.5 $\pm$ 3.2***

Figure 1 Time course of the induction of iNOS in the presence of glucagon and dexamethasone

Cells were incubated as described in Table 1 and samples were removed for the determination of $\text{NO}_3^- + \text{NO}_2^-$ production at the times indicated. Symbols: ○, plus cytokines; ●, plus cytokines and 1 μM glucagon; □, plus cytokines and 100 nM dexamethasone; ■, plus cytokines, 100 nM dexamethasone and 1 μM glucagon.

Figure 2 Effect of addition of glucagon on the induction of iNOS in the presence of dexamethasone and insulin

The experimental protocol was as described in the legend to Table 1. Dexamethasone (D), glucagon (G) and insulin (I) were added to final concentrations of 100 nM, 1 μM and 1 μM respectively. Control incubations (CON) in the absence of cytokines failed to show any detectable induction in the presence or absence of any of the hormones tested. The blot is representative of six different cell preparations.

these experiments is in good agreement with previous measurements of iNOS activity in liver by either the conversion of L-[U- ^{14}C]arginine to citrulline [23] or spectrophotometric methods [26,27]. Insulin (1 μM) had no significant effect on the induction of iNOS, in either the absence or presence of the cytokines. In contrast, the presence of a sub-optimal dose of 100 nM dexamethasone resulted in a 33% inhibition of NO production over the 21 h period as measured by the accumulation of both $\text{NO}_3^- + \text{NO}_2^-$ and a 31% inhibition of iNOS activity at the end of the experimental period. This agrees with the well-documented inhibition of iNOS induction by steroid hormones [3,26]. Inclusion of 1 μM glucagon during the induction period significantly inhibited $\text{NO}_3^- + \text{NO}_2^-$ accumulation in the medium by LPS plus the cytokines by 80–82%, irrespective of the presence of insulin or dexamethasone. A similar inhibition of iNOS activity was also observed, the percentages of inhibition being 70%, 79% and 80% respectively for control, insulin-treated and dexamethasone-treated cells. This indicates that the effect of the glucagon is unlikely to be at the level of the provision of substrates or cofactors for iNOS. The time course for the induction of iNOS activity is shown in Figure 1. There was no

Figure 3 Correlation between NO synthase activity and attenuation of bands on Western blots

The Western blots from three of the experiments in Table 1 were scanned and the attenuation (shown as optical density) was calculated as a percentage of the maximal attenuation obtained in the presence of the complete cytokine mixture in the absence of other hormones for each experiment. Similarly the NO synthase activity was determined as a percentage of the activity measured in the presence of the complete cytokine mixture in the absence of other hormones in each individual experiment for the same three experiments.

significant increase in $\text{NO}_3^- + \text{NO}_2^-$ output detectable until between 6 and 9 h, consistent with induction of the enzyme within this time period.

To ensure that the effect of glucagon was at the level of iNOS expression rather than any post-translational modification such as phosphorylation, the amount of iNOS protein was measured by Western blotting. The results are shown in Figure 2. The Western blots show results consistent with the measurements of both NO_3^- and NO_2^- and measurements of iNOS activity. There was no significant difference in iNOS expression in the presence of insulin, but co-incubation of the LPS plus cytokines with dexamethasone resulted in a decreased level of protein. The level of iNOS expression in the control cells in the absence of cytokines was below the level of detection, and similarly in cells treated with dexamethasone or insulin (results not shown). In the presence of glucagon, the level of iNOS protein was markedly lowered under all conditions. Figure 3 shows the correlation between NO synthase activity and the attenuation of the bands on the Western blot from a number of experiments after normalization for the maximal NO synthase activity and optical density obtained in the presence of the complete cytokine mixture in the different experiments. It is evident that there is a good correlation between the measured enzyme activity and the amount of protein expressed, suggesting that the effect of glucagon is not by covalent modification of the iNOS protein but as a result of changes in expression of the protein.

Effect of time of addition of glucagon on the induction of iNOS

Table 2 shows the effect of time of addition of glucagon after the addition of the LPS plus cytokines on the ability of glucagon to decrease NO formation and iNOS activity. It is evident that if glucagon was added at any point during the first 3 h of the induction period, it was capable of maximally inhibiting iNOS activity and subsequent NO production, suggesting that it is acting at a late stage during the induction process rather than the initial interaction of the cytokines with their receptors. A significant inhibition of activity was still seen after 6 and 9 h, although the ability of glucagon to inhibit NO formation became progressively less, presumably because a substantial amount of

Table 2 Effect of time of addition of glucagon after the cytokines on the induction of iNOS

Cells were incubated in the presence of 100 nM dexamethasone plus IFN- γ (100 i.u./ml), TNF- α (500 i.u./ml), IL-1 β (100 i.u./ml) and LPS (10 μ g/ml) for 21 h. Glucagon was added at the indicated times after the cytokines to a final concentration of 1 μ M. Samples were removed from the medium after 21 h for the determination of NO $_3^-$ + NO $_2^-$ production and the cells were processed for the measurement of iNOS activity. The results are expressed as means $\pm$ S.E.M. for five different cell preparations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for significance of difference from the treatment in the absence of glucagon; ††† $P < 0.001$ for significance of difference from the control incubation without cytokine.

	Time of addition of glucagon (h)	NO $_3^-$ + NO $_2^-$ production (nmol/mg of protein)	iNOS activity (pmol/min per mg of protein)
No cytokines	—	29.7 $\pm$ 12.9	0.26 $\pm$ 0.25
No glucagon	—	274.7 $\pm$ 38.3†††	42.4 $\pm$ 2.1†††
Glucagon	0	67.3 $\pm$ 12.7**	8.0 $\pm$ 3.1***
	3	63.2 $\pm$ 7.1**	6.8 $\pm$ 1.3***
	6	142.7 $\pm$ 18.8**	11.8 $\pm$ 2.2***
	9	192.0 $\pm$ 24.7**	23.9 $\pm$ 5.2*

Lane	1	2	3	4	5	6	7	8	9
Cytokines	+	-	+	-	+	-	+	-	+
Time of addition of Glucagon	-	9	6	3	0				

Figure 4 Effect of time of addition of glucagon on the induction of iNOS

The cells were incubated as described in the legend to Table 2, in the presence (odd-numbered lanes) or absence (even-numbered lanes) of cytokines. Glucagon (1 μ M) was added at the times indicated after the addition of the cytokines and the incubation was terminated and Western blot analysis performed after 21 h. The blot is representative of five similar experiments.

Treatment	CK 0 min	CK 30 min	GLU 30 min + CK
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Figure 5 Effect of pretreatment of cells with glucagon on iNOS expression

Cells were either pretreated with glucagon (1 μ M) or medium alone for 30 min; the medium was then removed and replaced with fresh medium in the absence of glucagon but in the presence of the LPS/cytokine mixture (CK 30 min, GLU + CK 30 min). The cells were then left for a further 21 h to induce iNOS. Also shown is a sample in which the cells were incubated with cytokines immediately without a change in medium (CK 0 min).

the total iNOS was expressed by 9 h. Figure 4 shows a typical Western blot of iNOS protein confirming that post-cytokine treatment of the cells with glucagon prevented the accumulation of iNOS up to 9 h after the addition of the cytokines. Figure 5

Figure 6 Effect of different concentrations of glucagon on the cytokine-induced formation of NO $_3^-$ + NO $_2^-$ and iNOS protein

The cells were incubated in the presence of the LPS plus cytokines for 21 h with or without glucagon at the indicated concentrations. At the end of the incubation period the medium was removed for the determination of NO $_3^-$ + NO $_2^-$ and the cells were processed for Western blotting. The results shown are typical of four separate cell incubations.

shows the effect of addition of glucagon to the cells for 30 min before treatment with the cytokines followed by removal of the glucagon. It is evident that a short exposure to glucagon was capable of blocking iNOS expression over the next 21 h, indicating that the effect of glucagon was rapid, long-lived and not readily reversible.

Dose-response relationship between glucagon concentration and the inhibition of NO production and iNOS protein

Figure 6 shows the effect of increasing glucagon concentration on iNOS protein and both NO $_3^-$ and NO $_2^-$ formation. It is evident that glucagon was capable of inhibiting iNOS expression and NO formation over the range 100 pM to 1 μ M. A half-maximal response was obtained at 3.7 ± 1.0 nM glucagon ($n = 4$).

Effect of dibutyl cAMP and forskolin on the induction of iNOS by LPS plus cytokines

As cAMP has been shown to synergize with cytokines to induce iNOS in vascular smooth muscle cells [11–14], rat renal mesangial cells [15–17] and cardiac myocytes [18], it was important to determine whether the effect of glucagon was via a cAMP-mediated mechanism. Table 3 shows the effect of the addition of 1 μ M dibutyl cAMP during the induction period with cytokines on NO $_3^-$ + NO $_2^-$ output and iNOS activity. Inclusion of dibutyl cAMP resulted in an inhibition of both NO formation and iNOS activity comparable to that of glucagon (Table 1), the percentages of inhibition being 78 % and 74 % respectively for NO $_3^-$ + NO $_2^-$ output and the conversion of L-[U- 14 C]arginine into citrulline. This conflicts with the ability of cAMP and analogues to synergize with cytokines in other tissues; however, it suggests that liver responds like cultured astrocytes in which noradrenaline and

Table 3 Effect of dibutyryl cAMP on the induction of iNOS

Cells were cultured as described in the legend to Table 1. Where appropriate, dibutyryl cAMP was added simultaneously with the cytokines to a final concentration of 1 μ M. The results are expressed as means $\pm$ S.E.M. for five or six different cell preparations. The measurements for the cytokine-treated cells in the absence of dibutyryl cAMP were significantly different from their appropriate controls at the level of at least $P < 0.01$. * $P < 0.05$, *** $P < 0.001$ for significance of difference from the appropriate treatment in the absence of dibutyryl cAMP.

Treatment	NO ₃ ⁻ + NO ₂ ⁻ production (nmol/mg of protein)		iNOS activity (pmol/min per mg of protein)	
	— Cytokines	+ Cytokines	— Cytokines	+ Cytokines
Control	15.1 $\pm$ 7.3	350.2 $\pm$ 36.7	3.3 $\pm$ 0.6	37.9 $\pm$ 4.7
Dibutyryl cAMP	7.6 $\pm$ 2.6	78.4 $\pm$ 23.2***	1.8 $\pm$ 0.6	10.0 $\pm$ 4.3*

Figure 7 Effect of different concentrations of dibutyryl cAMP on the cytokine-induced formation of NO₂⁻ + NO₃⁻ and iNOS protein

The cells were incubated in the presence of the LPS plus cytokines for 21 h with or without dibutyryl cAMP at the indicated concentrations. At the end of the incubation period the medium was removed for the determination of NO₂⁻ + NO₃⁻ and the cells were processed for Western blotting. The results shown are typical of three separate cell incubations.

dibutyryl cAMP inhibit LPS-induced iNOS expression [19]. Figure 7 shows a typical dose-response relationship between the concentration of dibutyryl cAMP and either NO₃⁻ + NO₂⁻ formation or iNOS protein as measured by Western blotting. The cyclic nucleotide was effective over the range 30 nM to 1 μ M, with a half-maximal effect occurring at 170 $\pm$ 10 nM ($n = 3$) dibutyryl cAMP.

Figure 8 shows a comparison of the ability of glucagon, dibutyryl cAMP and 100 μ M forskolin to prevent iNOS expression. It is evident that all three agents significantly decreased the total amount of iNOS protein within the cells, confirming a direct cAMP-mediated inhibition of total iNOS protein and activity.

Figure 8 Comparison of the inhibition of iNOS expression by 1 μ M glucagon, 1 μ M dibutyryl cAMP and 100 μ M forskolin

The cells were treated with the LPS/cytokine mixture for 21 h in the presence of glucagon (GLU), dibutyryl cAMP (DBcAMP) or forskolin (FORSK) and the samples were processed for Western blotting. None of the agents had any effect on iNOS expression in the absence of the cytokines (results not shown). The blot is representative of three different cell preparations.

Conclusions

From the results presented above, it is evident that a mixture of LPS plus IFN- γ , TNF- α and IL-1 β resulted in the induction of iNOS activity in cultured hepatocytes. The presence of insulin had no significant effect on iNOS either at the level of flux through the enzyme, activity of the isolated enzyme or total protein expressed. In contrast, co-administration of glucagon together with the cytokine mixture inhibited all three parameters, the major effect being at the level of the total amount of protein, rather than alterations in substrate supply or covalent modification of the existing protein. The physiological significance of the effect of glucagon remains to be established but it is interesting to note that starved animals are more resistant to the effects of endotoxin [28] and therefore the elevated glucagon levels observed in sepsis and endotoxaemia [5–9] might act together with increased glucocorticoid levels to limit NO production within the liver. The effect was rapid in onset and long-lived, a 30 min pretreatment period protecting the cells from the induction of NO synthesis over the next 21 h in the presence of the cytokines. Similarly the addition of glucagon at any time within the first 3 h of cytokine treatment greatly decreased the effect of the cytokines, with a significant inhibition still being apparent when glucagon was added 9 h after the addition of the cytokines. The effect of glucagon was mediated via cAMP, suggesting that the cytokine signalling pathway in liver might be similar to that in cultured rat astrocytes [19], whereas the cytokines might be acting via a different signalling mechanism in vascular smooth-muscle cells [11–14], rat renal mesangial cells [15–17] and cardiac myocytes [18], where cAMP synergizes with cytokines to enhance iNOS expression and activity.

Note added in proof (received 7 July 1997)

After the acceptance of this paper, similar data were published [29].

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