ANNA B. WOJTCZAK, KONRAD S. FAMULSKI, MARIA BUSZKOWSKA and PAWEŁ LATOS

EFFECT OF GLUCAGON AND PHORBOL MYRISTATE ACETATE ON OXIDATIVE DEMETHYLATION AND LIPID PEROXIDATION IN ISOLATED HEPATOCYTES*

Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, Poland

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Oxidative demethylation of aminopyrine and peroxidation of endogenous lipids induced by cumene hydroperoxide were studied in hepatocytes isolated from fed male rats. Glucagon and phorbol-12-myristate-13-acetate (PMA) inhibited both processes in the concentration-dependent manner. Pretreatment of hepatocytes with 1 μM glucagon decreased oxidative demethylation by 75% and had a much smaller effect on lipid peroxidation. Preincubation with 1 μM PMA inhibited both processes by 25-30%. Phosphorylation of three isoforms of cytochrome P-450 was observed in microsomes isolated from hepatocytes incubated in the presence of [32P]orthophosphate. After incubation with PMA the phosphorylation of all these proteins was increased by 60-100%, whereas glucagon increased the phosphorylation of only one isoform. Consequences of the phosphorylation of various isoforms of cytochrome P-450 for metabolic functions of the monooxygenase system are discussed.

The effect of hormones on the hepatic monooxygenase system was reported in early studies of Fouts [1] and Hutterer et al. [2] who found that catecholamines depressed hepatic metabolism of drugs and that injection of dibutyryl-cAMP decreased the content of cytochrome P-450. However, a detailed study on hormonal regulation of cytochrome P-450-dependent metabolic functions has been developed only in the last decade. It has been demonstrated [3] that cytochromes P-450 can be phosphorylated in isolated liver microsomes by cAMP-dependent protein kinase. Quite recently, phosphorylation of several isoenzymes of cytochrome P-450 has been observed in microsomes

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1 Abbreviations: CumOOH, cumene hydroperoxide; DTT, 1,4-dithiothreitol; PMA, phorbol-12-myristate-13-acetate; MDA, malondialdehyde; PMSF, phenylmethylsulfonyl fluoride.

2 The designation of cytochromes P-450 used in this study is according to Nebert et al. [30].
isolated from hepatocytes incubated with $^{32}$P orthophosphate [4-6]. Phosphorylation of purified isoenzymes of cytochrome P-450 by cAMP-dependent protein kinase and protein kinase C was also demonstrated [7-11]. It has also been documented that selective phosphorylation of cytochromes P-450 leads to a specific modulation of the monoxygenase activity [4, 9, 12, 13].

Apart from drug metabolism, peroxidation of hepatic endogenous phospholipids was shown to be related to the microsomal monoxygenase system and, in particular, the involvement of cytochrome P-450 in this process was documented [14]. The inhibitory effect of glucagon on peroxidation processes in isolated hepatocytes has been reported by Siess & Wieland [15]. However, the mechanism of this effect remains unclear so far and even the information on its subcellular localization is lacking. On the other hand, there is a great body of evidence that liver mitochondria isolated from glucagon-treated rats exhibit increased oxidative activity, ATP synthesis and other metabolic functions (for review see Ref. 16). Hence, it has been speculated [15, 16] that glucagon exerts a protective effect on mitochondrial integrity due to a depression of membrane lipid peroxidation in the intact cell.

In the present study we investigated the effect of glucagon and PMA$^1$ on isolated rat hepatocytes and found that both effectors decreased peroxidation of endogenous phospholipids and oxidative demethylation of aminopyrine. We also observed increased phosphorylation of some, not yet identified, isoenzymes of cytochrome P-450 in microsomes isolated from hepatocytes pretreated with glucagon or PMA.

**MATERIALS AND METHODS**

*Isolation and incubation of hepatocytes.* Fed male Wistar rats weighing 250-300 g were slightly anaesthetized with diethyl ether and livers preperfused with Ca$^{2+}$-free Hanks solution in open perfusion system. Then, livers were removed and perfused in recirculating system with Hanks solution containing 1 mM CaCl$_2$, 1% defatted and dialysed bovine serum albumin and 25 mg collagenase per 70 ml of the solution. Hepatocytes were isolated essentially according to Berry & Friend [17]. They were incubated at the concentration of 4-7 mg dry weight/ml in Krebs-Henseleit bicarbonate medium [18] containing 1 mM CaCl$_2$ and 1% defatted and dialysed bovine serum albumin under 95% O$_2$ + 5% CO$_2$ in a shaking bath at 37°C. Glucagon and PMA were added as 0.5 mM stock solutions in 10 mM HCl and dimethylsulfoxide, respectively. Final concentration of dimethylsulfoxide in the incubation medium did not exceed 0.2%. Duration of the incubations and other additions are specified in the legends to figures and tables. Incubation was terminated by the addition of perchloric acid to 5% final concentration, and 4-aminoanti-
pyrine was determined in neutralized extracts. For measurements of thiobarbituric acid - reactive products the incubation was stopped with trichloroacetic acid at 10% final concentration.

*Phosphorylation of microsomal proteins in isolated hepatocytes.* Hepatocytes isolated as described above were diluted to 10 mg dry weight/ml (corresponding to about $4 \times 10^6$ cells/ml) with the medium containing Krebs-Henseleit bicarbonate buffer without phosphate, 10 mM lactate, 1% bovine serum albumin and $[^32P]$orthophosphate 0.24 mCi/ml, carrier-free. Three 20 ml samples of this mixture were incubated for 30 min at 37°C in shaking bath under 95% O$_2$ + 5% CO$_2$. After 30 min one of the samples was supplemented with 1 μM glucagon and another one with 1.5 μM PMA (final concentrations), and the third one was left without additions (the control). The incubation was continued for 15 min. Thereafter, the samples were centrifuged for 2 min and the sedimented cells were washed once with cold Krebs-Henseleit buffer supplemented with 50 mM NaF, 0.2 mM PMSF and 1 mM EDTA. Washed cells were homogenized in a glass-teflon homogenizer in the medium containing 250 mM sucrose, 10 mM Tris/HCl buffer (pH 7.4), 1 mM EDTA, 50 mM NaF, 0.2 mM sodium orthovanadate and 0.2 mM PMSF. Microsomes were obtained by differential centrifugation and the microsomal pellet was immediately solubilized in the mixture consisting of 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 0.2 mM orthovanadate, 20 mM NaF, 0.2 mM PMSF, 20% glycerol and 0.6% sodium cholate. This final solubilize (1.0 ml) contained 10 mg protein (measured by the biuret reaction) and 9 nmoles of cytochrome P-450 measured according to Omura & Sato [19].

Solubilized microsomes were subjected to the discontinuous SDS-polyacrylamide gel electrophoresis (10% gel) according to Laemmli [20]. 50 μl of the solubilize was mixed with 20 μl of the solution of 130 mM KCl, 10% SDS, 2% mercaptoethanol, 20% glycerol and 0.01% bromophenol blue (pH 6.8). Samples were kept at room temperature for 15 min and applied on the gel.

*Chemical determinations.* 4-Aminoantipyrine was determined by the method of Trinder [21] as described by Bánhegyi et al. [22], using glucose oxidase, peroxidase and phenol. Corrections for zero time incubation were always made. Malondialdehyde, the product of peroxidation of endogenous phospholipids in hepatocytes and microsomes, was measured as thiobarbituric acid-reactive products as described by Buege & Aust [23], except that the samples deproteinized with trichloroacetic acid were centrifuged and the clear supernatant was heated with the TBA reagent at 100°C for 10 min. The amount of MDA was calculated based on its molar absorbance $ε_{535}$ of 156 cm$^2$/mM [23].

*Calculations.* Reaction rates in hepatocytes were expressed per g dry weight. They can be recalculated per g wet weight by dividing by a factor of 4.5. Statistical significance was evaluated using Student's $t$ unpaired test when comparing results from different cell preparations.
Chemicals. Collagenase from Clostridium histolyticum (grade II), glucose oxidase, peroxidase and ATP were from Boehringer (Mannheim, Germany); bovine serum albumin (fraction V), cumene hydroperoxide, phorbol-12- myristate-13-acetate and PMSF were obtained from Sigma (St. Louis, MO, U.S.A.); aminopyrine, glucagon and lactate were from Serva (Heidelberg, Germany); sucrose and inorganic salts were from Merck (Darmstadt, Germany). [32P]Orthophosphate was provided by the Institute of Nuclear Research in Świerk (Poland).

RESULTS

It is well known that organic peroxides, like t-butyl hydroperoxide and CumOOH, cause lipid peroxidation in biological membranes [14, 24, 25]. Figure 1 illustrates accumulation of MDA, the final lipid peroxidation product,

![Graph showing the dependence of MDA production on the concentration of CumOOH in isolated hepatocytes.](image)

Fig. 1. Dependence of MDA production on the concentration of CumOOH in isolated hepatocytes. Hepatocytes (4.8-5.2 mg dry weight/ml) were incubated for 30 min in the Krebs-Henseleit medium containing, in addition, 10 mM lactate and CumOOH at concentrations indicated. The results for MDA (▲) are means ± S.D. for 6 hepatocyte preparations or means for 2 preparations. Leakage of lactate dehydrogenase (LDH; mean values for two experiments) is expressed as percentage of the total activity in the cells which amounted to 938 U/g dry weight (○) during incubation of isolated rat hepatocytes with various concentrations of CumOOH. It shows that peroxidation strongly increased at CumOOH concentrations exceeding 200 μM and that this process ran parallel to an increased leakage of lactate dehydrogenase (EC 1.1.1.27) from the cells. Under these conditions the viability of the cells, as tested with Trypan blue, was considerably reduced after 20 min incubation.
The rate of lipid peroxidation by CumOOH also depended on the cell concentration; namely, it was increased and became less reproducible when the hepatocyte concentration was decreased below 2 mg dry weight/ml and was strongly decreased above 10 mg dry weight/ml. In the latter case, added CumOOH was most likely rapidly reduced by intracellular glutathione and therefore became ineffective. For this reason, in further experiments hepatocyte concentration was maintained within the limits of 4-8 mg dry weight/ml and the concentration of CumOOH never exceeded 180 $\mu$M.

Figure 2 shows that lipid peroxidation produced by CumOOH is diminished by glucagon and even more so by PMA. The spontaneous peroxidation
was hardly influenced by glucagon and only slightly by PMA (not shown). Concentration dependence of these effects of glucagon and PMA is shown in Fig. 3. PMA at 1 μM concentration reduced the peroxidation caused by CumOOH by about 30% and at 10 μM concentration by 70%. Glucagon was less effective and diminished MDA production only by 20% at 10 μM concentration.

Weiss & Estabrook [14] have suggested that cytochrome P-450 may be involved in microsomal peroxidation processes produced by CumOOH. It seemed therefore likely that the inhibition by glucagon and PMA of lipid peroxidation in hepatocytes as illustrated in Figs. 2 and 3 may be due to an effect of these substances on the microsomal monooxygenase system. To check
this hypothesis we followed oxidative N-demethylation of aminopyrine measured as accumulation 4-aminoantipyrine. As shown in Fig. 3, 1 μM glucagon produced a strong diminution of the demethylation by about 80%, whereas 1 μM PMA by about 20% only.

When lipid peroxidation and aminopyrine demethylation were measured in the same sample, both CumOOH and aminopyrine being present together in the same incubation, a competition between peroxidation and demethylation was observed. This was manifested by a significant decrease of MDA production by aminopyrine in both the absence and presence of lactate (Table 1). This is similar to the results obtained with isolated microsomes [14].

Table 1

Effect of glucagon and PMA on the demethylation of aminopyrine and the production of MDA in isolated hepatocytes

Hepatocytes (4-6 mg dry weight/ml) were preincubated in the Krebs-Henseleit medium for 5 min with 1.2 μM glucagon or 1.3 μM PMA in the absence or presence of 10 mM lactate and the reaction was started by addition of 160 μM CumOOH (final concentration). Where indicated, 1 mM aminopyrine was added simultaneously. The incubation was continued for 30 min and the reaction products were determined as described under Materials and Methods. All values are means ± S.E. for the number of cell preparations given in parentheses. The significance of the effects of PMA and glucagon, calculated by the Student’s t test, was: *, p < 0.05; **, p < 0.02; ***, p < 0.001

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<th>Lactate + Cumene hydroperoxide</th>
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<td>+ Aminopyrine</td>
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<td>MDA nmol/min per g dry weight</td>
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<td>53 ± 11 (6)</td>
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<td>18 ± 4** (4)</td>
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<td>190 ± 42 (4)</td>
<td>105 ± 15 (4)</td>
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<td>76 ± 5** (3)</td>
<td>90 ± 7** (4)</td>
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<tr>
<td>4-Aminoantipyrine nmol/min per g dry weight</td>
<td>710 ± 146 (8)</td>
<td>479 ± 99* (3)</td>
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<td>%</td>
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<td>410 ± 69 (4)</td>
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<td>304 ± 48*** (7)</td>
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Oxidative demethylation was strongly favoured in the presence of lactate whereas peroxidation was four times higher when CumOOH was used alone.

Both demethylation and lipid peroxidation were decreased by glucagon and PMA, the percentage of inhibition being the same in the presence and absence of lactate (Table 1). It is remarkable that the two effectors differ in their effect on demethylation and peroxidation, the former being more strongly inhibited by glucagon whereas the latter by PMA. A similar dependence is also evident from Fig. 3.
It is known that 4-aminoantipyrine can be acetylated in perfused liver [26]. Therefore, the question arose of whether the glucagon effect observed in isolated hepatocytes might be due to an increase of acetylated 4-aminoantipyrine, since it was not known whether the method of Trinder [21] estimated only free or also the acetylated form. This was checked by applying the method of Brodie & Axelrod [27] which differentiates between free and acetylated 4-aminoantipyrine. It was found that the contribution of acetylated 4-aminoantipyrine (about 20% of the total) was not affected by glucagon and PMA. Therefore, we could conclude that the inhibition of the oxidative demethylation of aminopyrine by glucagon and PMA was not a methodical artifact.

Since glucagon and PMA are known to stimulate the activity of protein kinases, further studies were undertaken to see whether the observed effects of these agents on lipid peroxidation and aminopyrine demethylation are correlated with the degree of cytochrome P-450 phosphorylation. The pattern of phosphorylated microsomal proteins from hepatocytes incubated with $[^{32}P]$phosphate is shown in Fig. 4. Microsomes from control hepatocytes exhibit three distinct phosphorylated protein bands in the region of cytochromes P-450 (50-55 kDa; Fig. 4B). In hepatocytes incubated with PMA the phosphorylation of these proteins was increased by 60-100% as documented for three experiments by cutting out and counting the whole 50-55 kDa region. A distinctly increased phosphorylation of only one protein was visible in microsomes from hepatocytes treated with glucagon. Although a precise identification of the three phosphorylated bands with different isoenzymes of cytochrome P-450 is difficult in the electrophoregrams obtained, we made an attempt in this direction based on their known electrophoretic location [4, 28, 29]. Thus, the most intensively phosphorylated band from glucagon-treated hepatocytes may be tentatively ascribed to P-450 IIb1 and corresponds to a faint protein band visible at this position (Fig. 4A). One of the lower bands may correspond to cytochrome P-450 IIC11 which is the most abundant in microsomes from uninduced rats as visualized by Coomassie stain (Fig. 4A).

DISCUSSION

The effect of glucagon and PMA on demethylation and lipid peroxidation

The known protective effect of glucagon treatment on mitochondrial functions [16] together with the observation [15] that glucagon diminishes lipid peroxidation in isolated hepatocytes prompted us to investigate a possible link between these two effects. Both demethylation and lipid peroxidation are catalyzed by the monooxygenase system [14]. Inhibition by dibutyryl-cAMP of aminopyrine demethylation in isolated mouse hepatocytes has been observed [12]. In our previous report [31] it has also been shown that glucagon decreases aminopyrine demethylation in isolated rat hepatocytes. It seemed
Fig. 4. Phosphorylation of microsomal proteins from isolated hepatocytes. Hepatocytes were incubated with [32P]orthophosphate with glucagon or PMA as described under Materials and Methods. Microsomes were solubilized and analyzed for 32P-labelled proteins by SDS-poly-acrylamide gel electrophoresis and autoradiography. A, Coomassie blue stain: lanes a and b, 140 μg and 70 μg microsomal protein per lane, respectively; lane P-450 represents partially purified preparation of isoforms IIB1 (●) and IIC11 (○). Molecular mass markers (M.W.) were: bovine serum albumin 66 kDa, ovalbumin 45 kDa and carbonic anhydrase 29 kDa. B, Autoradiogram of the same gel (2-day exposure to X-ray film). The region of cytochromes P-450 is marked with a bar; isoforms tentatively identified as IIB1 and IIC11 are marked as 1 and 2, respectively; the third band (marked 3) remains uncertain. This experiment was repeated three times with similar results.
therefore tempting to speculate that the monooxygenase system presents a possible common target for the hormonal effect on both lipid peroxidation and oxidative demethylation.

Phosphorylation of cytochrome P-450, in particular of the isoenzymes induced by phenobarbital, was observed independently in several laboratories [4-6]. This phosphorylation was reported to be catalyzed by cAMP-dependent protein kinase [4-6, 32] as well as by protein kinase C [9] and to be correlated with a decreased activity of the monooxygenase system [4, 9, 12, 13]. Our observation that not only oxidative demethylation but also lipid peroxidation are inhibited by glucagon and PMA leads to the assumption that phosphorylation of some cytochromes P-450 is the common mechanism involved in this hormonal action. Recently, Mkrtchian & Andersson [33] have reported that in vitro phosphorylation of microsomal proteins decreases the deethylation of ethoxyxoumarin but increases peroxidation of lipids. The latter effect is contrary to our results. However, these authors studied NADPH-dependent lipid peroxidation, whereas in the present study the peroxidation was produced by cumene hydroperoxide. It has to be stressed at this point that the mechanism of lipid peroxidation induced by CumOOH is quite different from that induced by NADPH. In the homolytic cleavage of CumOOH an extremely reactive cumyloxy-radical is generated, resulting in a partial destruction of cytochrome P-450 [14, 34]. As observed recently by Jansson et al. [35], phosphorylation of some isofoms of cytochrome P-450 further enhances their destruction. This can explain the effect of glucagon and PMA on CumOOH-induced lipid peroxidation as observed in the present study (Table 1 and Fig. 3). In contrast, NADPH-induced lipid peroxidation (“spontaneous” peroxidation) does not result in the destruction of cytochrome P-450, and phosphorylation of P-450 impairs its ability to interact with cytochrome b5 [10]. This is compatible with the observations of Mkrtchian & Andersson [33] and also with the lack of a substantial effect of glucagon and PMA on the spontaneous lipid peroxidation, as described in the present paper. On the other hand, demethylation of aminopyrine was inhibited by glucagon and PMA even in the absence of CumOOH (Fig. 3), which is in agreement with the results of Jansson et al. [10] and Mkrtchian & Andersson [33].

Phosphorylation of cytochromes P-450

In contrast to other authors [4-6], the present study was performed on hepatocytes from uninduced rats because the protective effect of glucagon on mitochondrial functions has been observed in such uninduced animals. The single significantly phosphorylated protein band in the region of cytochromes P-450 in microsomes isolated from glucagon-treated cells (Fig. 4) corresponds, most likely, to cytochromes P-450 IIB1 and/or P-450 IIB2, the main phosphorylated isoenzymes identified in glucagon-treated cells from pheno-
barbital-induced rats [4, 6]. On the other hand, Oesch-Bartlomowicz & Oesch [32] observed 4 phosphoprotein bands of cytochrome P-450 isoenzymes after cAMP treatment of hepatocytes. Two of these bands were ascribed to P-450 IIB1 and IIB2 whereas the other two were not identified. The phosphoprotein of microsomal cytochromes P-450 from hepatocytes after PMA treatment reveals 3 bands (Fig. 4). This pattern resembles those obtained in microsomes from liver of phenobarbital-induced rats treated with glucagon in vivo [4]. Although Koch & Waxman [4] and Pyerin & Taniguchi [6] failed to find a significant phosphorylation of cytochrome P-450 IIC11 after glucagon treatment, this possibility is not excluded in our experiments with PMA. Phosphorylation of purified cytochrome P-450 IIC11 by protein kinase C has already been described [9]. The aim of our further studies will be therefore identification of phosphorylated isoenzymes of cytochrome P-450 after PMA treatment of isolated hepatocytes.

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