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METFORMIN INDUCES AN AGONIST-SPECIFIC INCREASE IN ALBUMIN PRODUCTION BY PRIMARY CULTURED RAT HEPATOCYTES

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Abstract—Metformin (MET) is known to increase several biological effects of insulin (INS), but there is no information concerning its direct effects on protein synthesis. We studied the action of MET on albumin production by primary cultures of freshly isolated rat hepatocytes, alone or in combination with various agonists: INS, IGF-1, EGF, thyroxin, and dexamethasone. While having no effect alone, MET *in vitro* potentiates the effects of INS, IGF-1, and EGF. When this increasing effect toward INS was studied over a broad concentration range, MET appeared to improve low-acting INS levels and to intensify the maximal INS effects. In contrast, MET did not change the production of albumin stimulated by thyroxin or dexamethasone.

Animals chronically pretreated with MET *in vivo* showed a higher yield of isolated hepatocytes, better attachment, and especially higher viability after liver perfusion and during cell culture. This may largely explain why basal albumin rates were higher than in *in vitro*-treated cells. The effect of MET in the presence of the agonists exhibited the same agonist-specificity as *in vitro*.

Our data provide new insights into the pharmacology of MET by showing that hepatic protein synthesis is increased by MET and INS. From the specificity of action of MET towards INS, IGF-1, and EGF (but not thyroxin or dexamethasone), we hypothesize that this biguanide may act on intracellular pathways located between membrane receptors and sites of branching in the signaling cascades shared by these agonists.

Key words: hepatocyte; albumin; metformin; insulin; IGF-1; EGF; thyroxin; dexamethasone

The antidiabetic biguanide MET§ has been investigated in numerous experimental and clinical studies of the pathophysiology of diabetes mellitus. The efficacy of metformin was found to be dependent on the presence of insulin (INS) [1], suggesting that one major effect of this compound was to act synergistically with the hormone. It was shown that this action was due to yet undefined post receptor mechanisms of action. Thus, at least *in vitro*, MET increased glucose transport in insulin-sensitive tissues such as adipocytes [2, 3] and skeletal muscle [4]; this effect was shown to be related to the translocation of GLUT-4 in adipocytes [3]. MET was also shown to increase insulin-induced lipogenesis *in vitro* [5]. Another example of the potentiation of a biological effect of INS by MET was the maturation of *Xenopus* oocytes [6, 7].

Taken together, these data suggest that the capacity of MET to increase the cellular effects of INS is probably not limited to the transport of glucose. However, the effects of MET on protein synthesis, another biological process stimulated by INS [8], have never been investigated directly. We therefore studied the action of MET on the production of albumin by primary cultures of freshly isolated rat hepatocytes after either *in vitro* addition of the drug or previous *in vivo* chronic treatment of the animals.

Moreover, because MET has been shown to increase a series of biological effects selectively when induced by INS but not by other stimuli [9, 10], we aimed to test the drug's specificity in our model by comparing its effect on the albumin production stimulated by various agonists: insulin, insulin-like growth factor (IGF-1), epidermal growth factor (EGF), thyroxin, and dexamethasone, all known stimulators of this process.

MATERIALS AND METHODS

Materials

MET hydrochloride was a gift of LIPHA Labs (Lyon, France). Bovine insulin and IGF-1 were purchased from Boehringer-Mannheim (Meylan, France). Rat albumin specific antibodies and antigens were obtained from CAPPEL Kabs (Cochranville, PA, U.S.A.). Collagenase EGF, L-thyroxin and dexamethasone were purchased from Sigma (St Louis, MO, U.S.A.).

Isolation and culture of rat hepatocytes

Hepatocytes from male Wistar rats (IFFA Credo, L'Arbresle, France) were isolated by an *in situ* liver perfusion using collagenase, according to a procedure described by Seglen [11] and modified by Williams *et al.* [12]. After estimation of cell viability by the peripheral refractoriness of intact cells in phase contrast microscopy and the trypan blue test, freshly isolated hepatocytes were washed in basal William's medium E (WME)

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§ Abbreviations: MET, metformin; INS, insulin; IGF-1, insulin-like growth factor 1; EGF, epidermal growth factor; WME, Williams' medium E; SF, serum free; PBS, phosphate buffered saline; Alb, albumin.

supplemented with 10% (v/v) fetal calf serum, 70 μM cortisol, 2 mM L-glutamine, 10 mM HEPES buffer, and 4 mM NaOH. They were then plated at a density of $0.5 \cdot 10^6$ cells per 50 mm plastic cell culture dishes in the medium described previously for cell attachment for 6 hours at 37°. Subsequently, hepatocytes were washed three times in serum- and cholesterol-free medium (SF-WME) containing 4 g/l bovine albumin fraction V (Sigma) as transporter for 7.8 μM of a mixture of free fatty acids [13], and then were transferred to the SF-WME supplemented with the various agonists alone or with MET. For each group of experiments, hepatocytes from three or four livers were used.

Concentration of albumin

Albumin secretion rates were determined in the culture medium by the ELISA method [14]. Immunotitration plates (Polylabo, Strasbourg, France) were coated with a solution of antirat albumin IgG (100 ng/ml in PBS) and incubated overnight at 4°. Plates were then washed with NaCl 8.7 g/l, Tween 20 (0.05% v/v), and H_2O for 1:1.

A 200 μl volume of reference antigen solution (rat albumin) as well as antigen samples were put into wells, and dishes were incubated for 2 hours at 37°. After washing, 200 μl of rat antialbumin specific IgG conjugated with horseradish peroxidase was added to each well. IgGs were dissolved in PBS-Tween 20 (0.5–1 μg/ml), and dishes were incubated for 90 min at 37°. Following a last washing, 200 μl of a solution consisting of 0.1 mg/ml O-phenylenediamine (OPD) and 1 μl/ml H_2O_2 (3%) in phosphate buffer (0.01 M; pH: 6.0) was added to each well. Peroxidase catalyzes the polymerization of OPD into a chromophoreous compound that can be detected spectrophotometrically at 490 nm; the enzymatic reaction is blocked by adding 50 μl of 2.5 mM H_2SO_4/well.

Determination of cell viability during culture

After thorough removal of the incubation medium, cells were washed with PBS, but without Ca^{++} or Mg^{++}, and fixed for 10 min at room temperature with 10% formaldehyde (v/v) in PBS. After three washings with a borate buffer (10 mM; pH: 8.4), the cells were coloured with methylene blue (1% w/v in borate buffer) for 10 min. Water rinsing was followed by drying, and the fixed methylene blue was eluted by 0.1 N HCl for 15 min at 50°, according to Olsson *et al.* [15]. Then, 200 μl samples were put onto a microtitration plate and optical density measured at 630 nm by a Micro ELISA Autoreader MR580 (Dynatech).

Drug treatments

In vitro, MET was added in a dose range of 6 nM to 0.6 mM to test its effect in conjunction with INS. In subsequent experiments, the *in vitro* concentration of MET was fixed at 6 μM. *In vivo,* rats were treated daily with 50 mg/kg MET by oral route for 2 weeks. Without other specifications, the following agonist concentrations were used *in vitro:* Insulin = 0.1 μM; IGF-1 = 1.3 nM; EGF = 16 pM; thyroxin = 64 nM; and dexamethasone = 10 nM. These doses were found to be the most accurate in previous pilot dose-response experiments.

Statistics

Significances were calculated using Student's *t*-test. Values are expressed as mean ± *SD*.

RESULTS

In vitro *metformin experiments*

Metformin. MET added alone in a concentration range of 6 nM to 0.6 mM was without any increasing or decreasing effect on albumin production. These data, therefore, rule out any direct effect of the drug on this parameter.

Insulin. The data presented in Fig. 1 (open columns) show that INS exerted a dose-dependent stimulation on albumin production by the primary cultures of rat hepatocytes. This effect reached a plateau at 10 nM, since higher hormone concentrations did not further promote the amounts of albumin produced. When 6 μM MET was added to low-acting INS levels (1 nM), albumin induction rates were improved by 10–20% (Fig. 1, closed columns), indicating that MET had sensitized the hepatocytes to the effect of the hormone. At maximally acting INS levels, MET produced an additional 20–25% increase in albumin production, thereby revealing a potentiating effect of both substances.

IGF-1, EGF, thyroxin, and dexamethasone. Figure 2 includes the data related to these four agonists. After 18 h incubation, IGF-1 alone (1.3 nM) stimulated albumin production by 18% above control values, whereas addition of MET led to a 36% increase ($p < 0.01$). EGF (16 pM) induced a 57% increase above control, which was further augmented to 81% when coincubated with 6 μM MET ($p < 0.05$). Higher levels of EGF (1.6 nM) induced albumin levels 116% over controls, but could not be further extended by the addition of MET (data not shown).

Thyroxin (64 nM) increased albumin levels by 33% above control, and this amount was unchanged when 6 μM MET was added to the culture medium.

When dexamethasone was used as an agonist (10 nM), albumin production reached values 48% above control, which were not modified in the presence of additional MET.

In vivo *metformin treatment*

Effect of metformin. The treatment of normal rats for 2 weeks with 50 mg/kg/day oral MET had no effect on either body weight or glycemia.

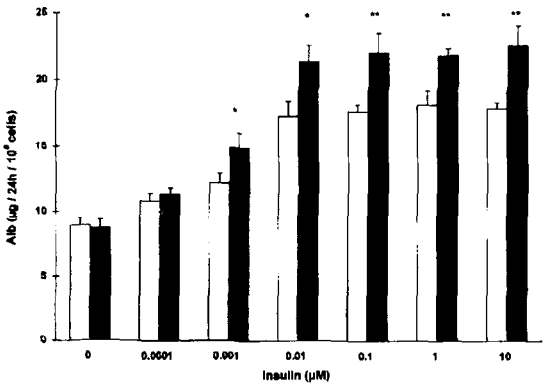

Fig. 1. Effect of a fixed concentration (6 μM) of MET on the dose-effect response of albumin production by cultured hepatocytes to increasing concentrations of insulin *in vitro.* Cells were cultured for 6 h at 37° in complete medium as described in Materials and Methods and incubated for 24 h at 37° in WME-SF containing insulin without (open columns) or with (black columns) metformin. $*P < 0.05$. $**P < 0.01$. $n = 3$ livers, 8 samples each.

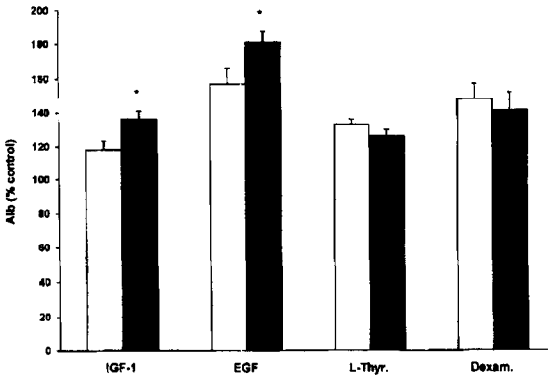

Fig. 2. Albumin production by hepatocytes after 18 h incubation with various agonists (IGF-1: 1.3 nM, EGF: 16 pM, L-Thyr: 64 nM, and Dexam: 10 nM) (open columns). Effect of metformin (MET, 6 μM), added *in vitro* to the incubation medium (black columns). Values are expressed as % of unstimulated control cells. $*p < 0.05$. $n = 4$ livers, 8 samples each.

However, the number of cells obtained after perfusion of the livers was significantly enhanced by 48–74%, as was the number of viable cells (above 90%). This better viability was maintained for the whole duration of the cultures; however, this effect was not observed in the previous experiments performed by the *in vitro* addition of MET alone (Table 1). We also observed that cells pretreated with MET *in vivo* attached much earlier and spread out better on the culture substratum (Fig. 3).

In contrast to the absence of drug effect in the pure *in vitro* studies, the improvement in cell viability was accompanied by increased albumin production during the first 18 hours of culture (Table 2) when using hepatocytes from *in vivo* pretreated rats.

Metformin and agonists. Figure 4 shows that the chronic *in vivo* treatment with MET led to a highly significant additional increase in albumin production when insulin, IGF-1, and EGF were used as agonists. However, as was the case in the *in vitro* study, no drug effect could be observed when either thyroxin or dexamethasone was used as agonist.

DISCUSSION

Protein synthesis as well as proteolysis are two main processes controlled by insulin. In particular, albumin synthesis is under tight control of this hormone [8, 16]. We observed that the *in vitro* addition of INS to primary cultured hepatocytes resulted in an increase in albumin production, which peaked at hormone concentrations of 10 nM, a value close to those reported in other studies [16]. IGF-1 is structurally very close to INS [17], as concluded from their sequence homology [18, 19], from the observation of possible hybrids formed by both receptor types [20], and from the discovery of common signaling pathways [21]. Although the existence of IGF-1 receptors in adult hepatocytes has been questioned [22], other studies have clearly confirmed that at least adult rat hepatocytes exhibit IGF-1 receptors [23, 24]. It has also been claimed that the effect of IGF-1 on liver cells could be mediated by the receptors for INS [25]. In accordance with other studies [18], we found that both hormones induced an increase in albumin synthesis, albeit somewhat smaller with IGF-1.

EGF is another well-known agonist of hepatic protein synthesis [26, 27] and, like INS or IGF-1, its cellular signaling pathways involve tyrosine kinases. Our data verified that EGF was indeed a very potent stimulator of hepatocyte albumin production.

In contrast, L-thyroxin and dexamethasone are agonists of albumin synthesis that do not use tyrosine kinase pathways [28–33]. In particular, thyroid hormones are known to bind preferentially to nuclear sites [34], and their binding to plasma membrane receptors is not related to clearcut biological effects [35]. Again we found that both substances strongly increased the albumin level.

Metformin is an oral antidiabetic that is dependent for most of its effects on the presence of insulin and one that acts at a postreceptor level [1]. In some studies, changes in insulin binding have been observed which could, however, not be automatically linked to the drug efficacy. Metformin did not modify insulin binding in liver cell lines [10]; therefore, this mechanism is unlikely to explain the effects of the drug in the present experiment. Metformin has been extensively studied for its various effects on insulin's biological functions; in addition to the well-known effects of MET on INS-stimulated glucose transport [3, 4, 11], GLUT-4 translocation [3], and glycogen synthesis [36, 37], it was also demonstrated to act synergistically with INS on hepatic gluconeogenesis [38], lipogenesis [5], and glucose incorporation into triglycerides [39], and to accelerate the INS-induced maturation of Xenopus oocytes [6, 7]. In contrast, few data exist concerning the effect of this drug on protein synthesis. *In vitro* we found no effect of MET alone on albumin production, whatever the dose used. Previous reports also failed to show an effect on the incorporation of amino acids into liver proteins [40] or uptake of amino acids into muscle L6 cells [41]. It also had no influence on basal, INS-stimulated, or glucagon-stimulated transport of AIB into cells, suggesting that an increase in protein uptake by cells does not explain our observations [42, 43]. In several studies, the addition of cycloheximide did not interfere with the effects of MET, suggesting that the drug did not induce protein synthesis in order to exert its effects on glucose transport [44,45].

Table 1. Rate of cell viability during culture of hepatocytes obtained from perfused livers of control and *in vivo* MET-treated rats (50 mg/kg/d for 2 weeks)

	Time after seeding			
	24 hours	48 hours	72 hours	96 hours
Control rats	49 ± 3	36 ± 2	23 ± 1	15 ± 1.5
In vivo MET-treated rats	86 ± 6*	57 + 3*	30 ± 4†	26 ± 6‡

For each time after seeding, the culture medium was removed and viable cell percentage performed by methylene blue method. ‡ $p < 0.05$. † $p < 0.01$. * $p < 0.005$.

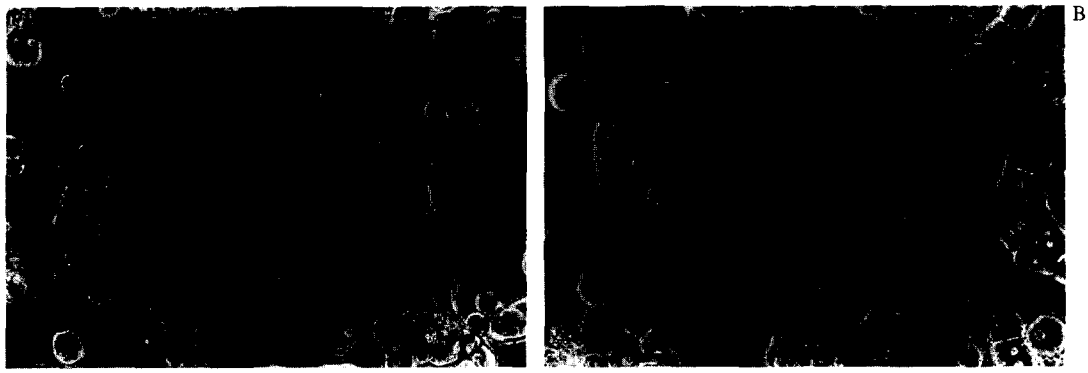

Fig. 3. Comparison of freshly cultured hepatocytes from untreated (A) and *in vivo* metformin-treated (B) rats 9 h after seeding. For each group of experiments, $0.5 \cdot 10^6$ cells were plated per 50 mm plastic cell culture dish in complete medium for 6 hours at 37° and transferred to the SF-WME. Photographs illustrate higher spreading of cells from *in vivo* treated animals, due to better adherence to the substratum.

Table 2. Effect of a chronic *in vivo* treatment of rats with MET (50 mg/kg/d for 2 weeks) on albumin production (ng/h/10^6 cells) by primary cultures of freshly isolated rat hepatocytes

	Time after seeding		
	6 hours	12 hours	18 hours
Control rats	229 ± 36	335 ± 13	419 ± 15
In vivo MET-treated rats	203 ± 30	441 ± 53*	602 ± 40*

Cells ($0.5 \cdot 10^6$/dish) were treated as described in Materials and Methods. Albumin concentrations were determined by ELISA and the number of cells estimated by methylene blue method.

* $p < 0.05$.

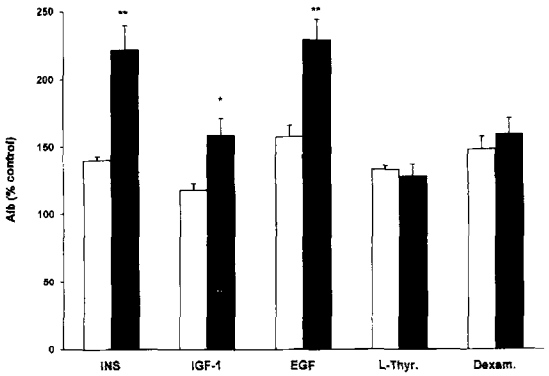

Fig. 4. Albumin production by primary cultures of rat hepatocytes after 18 h incubation with various agonists (INS: 0, 1 μM, IGF-1: 1.3 nM, EGF: 16 pM, L-Thyr: 64 nM, and Dexam: 10 nM) in untreated (open columns) or chronically *in vivo* MET-treated rats (black columns). Values are expressed as % of unstimulated control cells. *$p < 0.05$. **$p < 0.01$. $n = 4$ livers, 8 samples each.

In a study on cells from rabbit aortic media, MET even decreased protein synthesis [46], as did other biguanides in liver cells [47, 48].

There is therefore no reason to suggest that MET could directly favour protein synthesis. Nevertheless, when a fixed dose of MET was used (6 μM), an additional 25% increase in albumin production was observed, one found even at maximally acting INS levels. From these studies, we infer that MET acts in an additive way to INS, rather than by potentiating the hormone's effect directly. This conclusion is supported by similar findings in adipocytes [3, 39], and suggests that MET most likely acts on intracellular biochemical steps common to those used by the hormone.

An increasing effect of MET was found with three agonists (INS, IGF-1, and EGF), but not with thyroxine or dexamethasone, thus showing agonist specificity. In contrast to INS, maximal EGF effects were not further potentiated by MET. This can be explained by our observation that, of all groups, EGF at 16 nM was the most potent stimulant of albumin synthesis, and that the synthetic capacity of the cells may likely have been saturated. Such effects have been reported in other experimental situations. Oocyte maturation was only increased by MET when stimulated with INS, but not with progesterone [9] or MGBG (Methyl Glyoxal-Bis[Guanylhydrasone]) [6]. Similarly, MET modulated PAI-1 production by HepG2 cells when added to INS, but not to phorbol esters or interleukin [10]. Our own data show that, although we cannot exclude that INS and IGF-1 may have acted through the same receptors, the fact that all three positive cases—but not the negative ones—belong to substances using tyrosine protein kinase pathways is an intriguing observation. It thus appears from all these data that the site of action of MET may be related to the tyrosine kinase-dependent pathways of hormone signaling. An effect on the receptor tyrosine kinase is unlikely, since mainly negative results were obtained in corresponding studies [49].

Metformin could also have increased the albumin production of the hepatocytes by stimulating an exocytotic process linked to the secretion of the protein into the extracellular medium. However, if this were the mechanism, the drug should have increased the albumin levels independently of the agonist used.

The chronic pretreatment of rats with a low MET concentration for 2 weeks did not modify body weight or plasma glucose levels. Using hepatocytes from these rats, we found the same agonist-dependent effects as in the *in vitro* study. The effects of hormones and dexamethasone on albumin production were more pronounced than in the *in vitro*-treated hepatocytes, but could be due to an influence of MET on the liver *in vivo*.

Indeed, the number of cells collected from each perfused liver was clearly higher. Even when comparing the albumin production rate for a similar number of cells, the higher levels obtained after *in vivo* treatment were probably a consequence of their better shapes after liver perfusion. In addition, the attachment of the cells to the substratum during the early hours post-seeding was clearly improved in *in vivo*-pretreated cells. We are aware of another study reporting such protective effects of MET on hepatocytes obtained from hypoxic rats as well as on erythrocytes exposed to osmolar stress [50]. Thus, MET could protect cell membranes against the stress that cells inevitably experience during isolation procedures. This tentative explanation would be supported by our own observations (Fig. 3) of more healthy-looking shapes of the hepatocytes from *in vivo*-treated rats. Alternatively, the higher yield of cells after liver perfusion could be due to a more homogeneous perfusion of the organ under chronic MET treatment. We are presently exploring this hypothesis, and preliminary observations indeed suggest that MET increases the nutritive microflow of the liver. An effect on liver blood flow had been previously described in rats treated with MET and submitted to oral glucose loads [51].

In conclusion, our experiments have provided new insights into the pharmacological properties of metformin. First they have demonstrated that, in addition to MET's improving effects on various biological functions of INS, protein synthesis is also potentiated by the association of both substances. Moreover, we believe that our data are an interesting contribution to the understanding of the mechanism whereby MET acts synergistically with INS. Indeed, considering our present results together with other data from the literature, we may infer from the specificity of the drug's effects that it acts at a postreceptor step most likely linked to tyrosine kinases, but located before the branching of individual signaling pathways of insulin.

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