MEMBRANE PHYSIOLOGY AS A BASIS FOR THE CELLULAR EFFECTS OF METFORMIN IN INSULIN RESISTANCE AND DIABETES

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SUMMARY - Many recent data provide new, original insights into the mechanisms of action of the antidiabetic Metformin. Careful selection of most relevant data in terms of dosage prompted this original review, largely devoted to the drug action at the cell level and whose hypotheses/conclusions are tentatively interpreted according to corresponding basic scientific knowledge. Metformin interferes with several processes linked to HGP (gluconeogenesis, glycogenolysis and their regulatory mechanisms), lowering glucose production and resensitizing the liver to insulin. The hepatic drug effect is largely favoured by prevailing glycemia. In peripheral tissues, metformin potentiates the effects of both hyperglycemia and hyperinsulinemia. Increase in glucose-mediated glucose transport is mainly mediated by an improvement in the glucose transporter's intrinsic activity. Potentiation of the hormone effect relates to an increase in insulin receptor tyrosine kinase activity. Both mechanisms (insulin signalling and glucose transport) result in the activation of glycogen synthase, a limiting enzyme in the causal defects of NIDDM. Exciting findings show that, conversely, priming cells with very low insulin concentrations also leads to full expression of metformin's antidiabetic activity. Specific investigations confirm a working hypothesis defining the site of action as the cell membrane level. Indeed metformin corrects membrane fluidity and protein configuration disturbed by the diabetic state and which interfere with normal protein-protein or protein-lipid interactions required for proper functioning of the processes regulating glucose transport/metabolism. It is proposed that membrane changes largely represent a common denominator explaining metformin effects on various systems involved in receptor signalling and related functions.

Key-words: metformin, diabetes, insulin resistance, glucose transporters, insulin receptor, tyrosine kinase, membrane fluidity.

Forty years after its first application as a therapeutic principle for treating non-insulin-dependent diabetes mellitus (NIDDM), metformin (MET) has revealed only some of the mysteries concerning its modes of action. This drug occupies a particular place in the treatment of Type 2 diabetes since it is the unique representative of the class of biguanides and has many characteristics which make it distinguishable from other therapeutic principles, such as sulphonylureas, thiazolidinediones or alpha-glucosidase inhibitors.

For many reasons, it is important to gain further insight into the pharmacology of this drug. Physicians have placed their confidence in it, and prescriptions need to be appropriate to the particular pathophysiological profile of each individual NIDDM patient at the time of disease discovery and, even more importantly, during the subsequent disease course. On the other hand, identification of the mechanisms whereby MET is able to counteract insulin resistance and lower hyperglycaemia also makes this drug a tool for improved understanding of fundamental aspects in animal and clinical investigations within a kind of feedback loop. Over the years, numerous reviews have documented the ongoing interest of clinicians, pharmacologists and basic researchers in understanding how MET counteracts insulin resistance and reduces hyperglycaemia [1-6]. Most of these papers have dealt with pharmacodynamics, tissue-organ pharmacology and clinical aspects.

The entirely new analysis presented here focuses on selected relevant findings at the cellular level, including recent cardinal investigations. It tentatively places these data in the light of our current knowledge on cell membrane biology in insulin signaling and glucose metabolism. By describing in detail the direct action of the drug on cells, it tries to discriminate direct effects from those resulting indirectly from improvement of the diabetic state. Finally, a plausible hypothesis on the cellular modes of action of MET is proposed.

**IMPORTANT PRELIMINARY CONSIDERATIONS**

Pharmacodynamic and pharmacokinetic characteristics of metformin - MET, a biguanide, is a weak base exhibiting a cationic charge at physiological pH and a pKa close to 12. The drug circulates in both bound and free form with no covalent binding. As indicated below, this has clearcut consequences on its behaviour in cell pharmacology.

A key point in this analysis is the relevance of drug concentrations used in many experimental approaches. The half-life of the major part of the drug is about 90 min, with peak peripheral plasma levels hardly exceeding $10^{-4}$ M after oral tablet intake and “fasting” plasma levels close to $10^{-3}$ M (about 1 µg/ml). Portal blood levels in rats peaked at $5.10^{-4}$ M [7]. Measurements of tissue levels in various animal species have shown that only gut, liver and kidney accumulate MET for some hours [7]. In the intestine, MET concentrations can amount to $10^{-2}$ M, but the exact location of the drug in the gut wall has not been satisfactorily determined [8, 9]. In the liver, drug concentrations are slightly above plasma levels, and in peripheral tissues such as skeletal muscle or fat, which are of particular interest in this pathology, MET concentrations are not very different from plasma levels.

The first conclusion, therefore, is that drug levels above 50 µg/ml for hepatocytes and 5 µg/ml for muscle or fat cells are not relevant. As will be seen below, this is of considerable importance.

Dose-effect relationships – When we review the immense set of data available on experimental pharmacology performed with MET, it becomes evident that it has many diverse biological effects, which are frequently typical for relatively narrow concentration ranges and which differ accordingly. Most importantly, there appears to be a very marked change in MET pharmacology at levels very close to $5.10^{-5}$ M. This is the level where direct biological effects appear, which at lower drug concentrations are absent or require the presence of insulin. Typical illustrations of this phenomenon are the direct inhibition of gluconeogenesis or the direct stimulation of glucose transport in skeletal muscle, cardiac or fat cells. These drug levels also correspond to the threshold of the non-toxic inhibition of cellular respiration by MET. In fact, no study has demonstrated direct inhibition of gluconeogenesis below $5.10^{-4}$ M, and even insulin only moderately shifted the dose-effect curve [10]. A series of studies performed on adipocytes, various types of muscle cells and other cell types showed the direct effects of MET on the translocation of glucose transporters above $10^{-3}$ M in the absence of insulin [11-13]. Such drug levels are not reached in humans even in cases of severe clinical situations [14]. As suggested [15], these effects are typical of Pasteur effects and therefore likely to concern the intracellular pool of glucose transporters involved in hypoxia and contraction [16]. Moreover, we have evidence (N.Wiernsperger, J. Rapin and P. Claudy, unpublished results) that at these concentrations MET tends to build up reversible intermolecular binding, which could confer other properties related to abrupt modifications in the drug’s pharmacological behaviour. Unfortunately, many investigations have dealt with such high doses, even until recently.

These considerations are of utmost importance - MET concentrations in tissues do not exceed about...
25 µg/ml (5.10^{-4} M) in liver and about 10 times less in other tissues relevant to insulin resistance/diabetes. Therefore, experiments performed in vitro with higher doses have no relevance and may even be misleading. Direct effects of the drug are either weak or absent at concentrations below 5.10^{-4} M and, with few exceptions, require the presence of insulin. Concentrations above 5.10^{-4} M exert sizeable direct effects on glucose transport and metabolism, but are linked to biochemical processes induced by the reduction of the ATP/ADP ratio (although the latter is not toxic for the cell). It is therefore crucial to realise that data originating from experiments using high MET concentrations are not only essentially irrelevant but have also led to erroneous results that have induced misleading conclusions. There is little doubt that this “historical” background has largely contributed to the confusion associated with interpretations of MET modes of action. For these reasons, subsequent analysis here will not consider such data, except when useful for a better understanding.

**FACTORS MODULATING METFORMIN ACTION**

A series of factors clearly influences MET pharmacology, which has to be considered carefully in interpreting data. As discussed above, direct effects of MET, i.e. in the absence of insulin, are usually weak or non-existent at therapeutic concentrations. With few exceptions, MET requires the presence of insulin or elevated glucose, or both, to show activity.

**Insulin** – While there is some evidence in support of the insulin-independent mechanisms of MET, these are probably qualitatively too limited to induce any significant effects resulting in a reduction in hyperglycaemia (see the next section). Thus, in insulin-free states such as Type I diabetes in rats, the effects of MET on hyperglycaemia cannot be seen until extremely high oral doses are administered. The requirement for insulin and the enhancement of insulin-related effects on drug addition has led to the concept of MET-potentiated hormonal effects. In fact, many data illustrate this phenomenon, and clinical experience shows that MET not only acts as an antidiabetic in NIDDM but clearly reduces the daily needs for insulin in insulin-dependent diabetes mellitus patients [17].

However, a closer look at the relationship between MET and insulin reveals a more complicated situation. Indeed, old observations in vivo showed that MET was able to lower hyperglycaemia in the presence of very low insulin concentrations [18]. Very recently, a reevaluation of this question has confirmed that insulin concentrations as low as to be barely detectable by standard analytical procedures were sufficient to observe the antidiabetic effect of MET in vivo [19]. In vitro, similar observations have been made in rat cardiomyocytes, where MET enhanced glucose transport in the presence of insulin concentrations which were low and totally inactive per se [20]. In the skeletal muscle of rats pretreated with MET for three weeks, glucose transport in vitro was enhanced in soleus and EDL muscles by insulin concentrations as low as 2 µU/ml, which are inactive alone (H.Bachelard, personal communication). Even more interesting, preincubation of red blood cells with a physiological concentration of insulin enhanced the activation of glucose transport in vitro by MET, although these cells are totally unresponsive to the hormone (Table I) [4].

Clearly, in this situation insulin has a priming effect on cell membrane and glucose transport, reminiscent of that reported for low hormone levels in insulin-sensitive cells [21]. These data are intriguing since they demonstrate clearcut biological effects in the presence of insulin concentrations which are unable to stimulate glucose transport per se. Therefore, they raise many questions about the nature of the relationship between both substances. They also suggest that MET and insulin act in a cooperative fashion, possibly by complementary actions on common biochemical pathways rather than by a true potentiation of the hormone. Obviously, insulin and MET have a reciprocal permissive action.

**Glucose** – It is a common observation that MET in vivo only reduces glycaemia if there is a supranormal blood sugar level. Several factors have been proposed to explain this phenomenon, for example, a net equilibrium between antagonistic effects that nullify each other. Similarly, many reports have shown that MET acted only or at least much better in hyperglycaemic than euglycaemic clamps [22-24]. However, this effect is also regularly observed in vitro, i.e. in situations where there is no compensatory system available. Thus, hyperglycaemic incubation media enhance MET-induced glucose transport in various cell types, such as skeletal muscle cells [25, 26], adipocytes [11].
red blood cells [27], lymphocytes [28] or vascular smooth muscle cells [13]. These data clarify one major mechanism of MET action, namely an increase in glucose-mediated glucose transport. Considering the importance of this phenomenon in established diabetes (mass action effect of glucose) as a substitute for defective insulin action [29, 30] and the evidence that this compensatory process also becomes limited in these patients [31], it is assumed that such a mode of MET action largely contributes to its overall beneficial action on hyperglycaemia. It might particularly apply to reduction of fasting hyperglycaemia, a period during which non-insulin-mediated glucose transport plays the most important role. Interestingly, the anti-gluconeogenic effect of MET is also more potent when hepatocytes are incubated with high glucose [32, 33].

Duration of incubation – A series of reports has shown a MET effect only after prolonged incubation [13, 34-36]. This was particularly true for in vitro studies on glucose transport, whereas the addition of the protein synthesis inhibitor cycloheximide had no effect on the results, which suggests that it was not due to de novo synthesis of proteins or glucose transporters [36]. However, other studies have shown that typical direct metabolic effects of MET did not require prolonged incubation (see below).

Cell status – Another frequent observation has been that MET effects can only be seen in organisms or cells which are insulin-resistant, even if there is no hyperglycaemia. Thus, MET in vitro inhibited G-6-Pase only in hepatocytes from diabetic rats [37]. Several stimulatory effects on insulin receptor kinetics were only seen in cholesterol-modified vs normal hepatocytes [38]. Typically, the addition of MET to human muscle biopsies induced an improvement in glucose transport only in samples from insulin-resistant patients, but had no effect in those obtained from normosensitive patients (Fig. 1) [39]. Similar observations have been made concerning non-invasive measurements in human liver [40]. When administered in vivo at normal doses, MET had no measurable effects on metabolic parameters of normal cells, while improving situations characterised by normoglycaemic insulin resistance [41-44].

**MAIN EFFECTS OF METFORMIN ON GLUCOSE TRANSPORT/METABOLISM**

Before considering in greater detail how MET may act, it is important to recall its main biological effects in the field of interest covered by this review, i.e. insulin resistance and diabetes. Actually, it has become clear from many investigations that MET has a dual effect against insulin resistance and NIDDM by acting at the level of both the liver and peripheral tissues (mainly skeletal muscle). Since many excellent recent reviews have covered the organ pharmacology of MET, only an overview is provided here concerning the parameters important for understanding the cellular mechanisms of MET action.

**Liver**

Most, although not all, clinical studies have shown that a main action of MET in NIDDM is to reduce hepatic glucose output [24, 25]. This is probably achieved by different mechanisms since it appears that
MET affects all major contributors to excessive hepatic glucose production. The antiguconeogenic effect of MET, for example, is the result of a variety of interference sites of the drug with the chain of events involved in gluconeogenesis. Recent studies have shown that MET reduces the hepatic transport of precursors such as lactate [46] or alanine [47] at concentrations found in portal vein blood after oral drug intake. To some extent, the antiguconeogenic effect of MET is reinforced when insulin is present [10, 33]. It could also act by decreasing peripheral free fatty acids (FFA) which stimulate hepatic gluconeogenesis. One main action of MET on the processes involved in HGP is an inhibition of the hepatic effects of glucagon [32, 33, 35, 47-49]. Treatment of obese Zucker rats with MET restored the reduction in adenylyl cyclase activity induced by insulin, increasing the Ki for the hormone. Conversely, MET also reduced the capacity of glucagon to increase this enzyme activity by decreasing the expression of a Gi protein [50, 51]. This action has been repeatedly demonstrated and is located at a step behind receptor binding since MET did not change plasma glucagon levels or inhibit glucagon binding [35]. This effect might largely explain the reduction in fasting hyperglycaemia, since the glucagon/insulin ratio is a major determinant of HGP in diabetic patients. In addition to inhibition of gluconeogenesis, the antigucone effect also reduces glycogenolysis. In cultured rat hepatocytes, MET reduced glycogenolysis induced by phenylephrine through inhibition of an IP3-modulated calcium channel [52]. Interestingly, this effect was also higher in the presence of insulin and related to a reduction in cAMP. Recent data also show that MET interferes with G-6Pase, a key enzyme in the metabolic handling of glucose and glycogen in the liver [37, 43].

Finally, the suggestion, resulting from several clinical studies, that MET sensitises the liver to insulin has recently gained support from an animal model of hepatic insulin resistance. Indeed high-fat fed rats rapidly exhibited resistance to HGP inhibition by insulin, and this effect was remarkably normalised at submaximal hormone levels by a chronic low-dose treatment with MET [43]. The fact that MET increases hepatic insulin receptor signaling was recently reported for rats. Preliminary data showed that insulin receptor kinase activity, IRS-2 phosphorylation and PI3kinase were increased shortly after an acute oral administration of MET [53]. Thus, MET has a broad spectrum of actions on various processes involved in HGP, since both direct insulin-independent and insulin-dependent pathways are influenced according to the actual metabolic state of the liver and the prevailing drug concentration.

Peripheral tissues

**Glucose transport** – MET itself does not directly increase glucose transport in conditions of normoglycaemia and absence of insulin, unless supratherapeutic concentrations are used. However, as discussed above, the latter data have little relevance in normal clinical situations. Typically, MET does not increase glucose transport in X.oocytes in the absence of insulin when added at concentrations corresponding to therapeutic plasma levels [54]. Hyperglycaemia favours MET stimulation of glucose transport, as seen in erythrocytes incubated in hyperglycaemic medium [27]. Since erythrocytes are cells equipped with only GLUT-1 transporters and are unable to synthesise proteins, these data strongly suggest that MET enhances the transport capacity of individual transporters and exerts a permanent action, even in periods when insulinemia is not elevated. In vitro and ex vivo measurements confirmed that MET also corrected glucose transport in red blood cells from diabetic or glucose-intolerant patients [27, 55, 56]. In human myocytes in vitro, a stimulation of glucose transport was observed above 50 µM MET, which increased when glucose was also elevated [26]. This effect was seen in the absence of insulin, but required 8-h incubation [25]. Since no difference was seen between measurements with either 3-OMG or 2-DG, it was concluded that transport rather than phosphorylation was the target of MET. A study using human mononuclear cells also found increased glucose uptake after prolonged incubation for as long as 14 h [57]. In cultured lymphocytes, MET increased glucose uptake above a concentration of 33 µM [28]. In a recent elegant approach performed in X.oocytes, it was shown that MET, at doses having no effect per se; strongly enhanced the effect of insulin, thus confirming the clinical observation at cellular level (Fig. 2) [54].

**Glucose transporters** – Considering the processes involved in cellular glucose transport, and depending on transporter subtypes, several mechanisms need to be considered to understand the action of this drug. Indeed, glucose transporters can be translocated from the cell interior to the plasma membrane and/or activated when inserted into the membrane. Even insulin itself is believed to exert a dual effect by favouring translocation, but also by increasing the intrinsic activity of membrane-located transporters (the latter possibly due to unmasking of inactive transporters). Moreover, although GLUT-1 are mainly located in the cell membrane (in contrast to GLUT-4 which are essentially intracellular), some GLUT-1 translocation occurs upon insulin stimulation. Therefore, drug effects on expression, translocation or activation of glucose transporters should be considered.

**Glucose transporter expression** – Several studies showing an increase of glucose transport by MET after prolonged incubation have reported similar data in the presence of cycloheximide, which suggests that
MET did not increase the expression of glucose transporters. All investigations performed on insulin-sensitive tissues playing a major role in insulin resistance/diabetes, i.e. fat and muscle, failed to show increased mRNA or protein levels after MET treatment [58-63]. Conversely, two positive findings relate to increased GLUT-1 (but not GLUT-2) in jejunum [64] and GLUT-1 in fibroblasts [65]. Thus, it does not appear that direct changes in transporter expression in insulin-sensitive tissues would be an important contributor to the mechanisms of MET action.

Glucose transporter translocation and recruitment – In vitro, GLUT-1 translocation has been observed in cultured human myotubes, whereas GLUT-4 were not affected [26, 36]. An effect on GLUT-1 and GLUT-3 was observed in human mononuclear cells [57]. In adipocytes in vitro, both GLUT-1 and GLUT-4 translocation was found at relatively high MET doses [60]. Other studies showing increased GLUT-4 translocation with MET have used supratherapeutic doses of the drug and are therefore not considered here. However, it is plausible – but not demonstrated – that a chronic treatment by MET sensitises cells by improving their state of insulin resistance, so that improved translocation might occur at therapeutic drug concentrations in vivo. Such data are largely missing at present, but results from chronically MET-treated Zucker rats or rats made insulin-resistant by dexamethasone do not lend support for a major effect of MET on GLUT-4 translocation [59, 63]. These data could mean that MET acts rather on GLUT-1, while insulin might primarily affect translocatable transporters such as GLUT-4, thus resulting in their global synergistic action.

Glucose transporter intrinsic activity – Alternatively, or additionally, MET could behave in an insulin-like fashion by unmasking inactive transporters located in the cell plasma membrane, either directly or by enhancing the hormone effect on this particular phenomenon [66]. In the absence of major effects of MET on expression or translocation of glucose transporters, changes in their intrinsic activity need to be considered. A variety of agonists, as observed with MET, induces quantitative effects which are above the values accounted for only by additionally recruited transporters. It is known that glucose transport is a two-step process resulting from recruitment to the plasma membrane (or the unmasking of inactive membrane-inserted ones), followed by a stimulation of their intrinsic activity [67, 68]. High insulin is linked to the low catalytic activity of GLUT-1 [69], possibly representing a mechanism whereby some compensation can be achieved in situations of impaired cellular response to insulin. Thus, the unmasking of reserve transporters or of their individual transport capacity appears to be a mechanism whereby MET could further increase transmembrane glucose transport stimulated either by glucose itself or insulin. Calculations of the quantitative effect of MET on glucose transport, as compared with the limited drug effect on transporter translocation, have suggested that MET might also increase their intrinsic activity [26, 60].

Fig. 2. Effect of MET ± insulin on glucose uptake in Xenopus oocytes (n = 4 in duplicate) (MET 20 µM) (INS 2 µU) (modified from 54)
Such an effect is corroborated by the observation of increased glucose uptake by MET in erythrocytes, i.e. cells lacking both transporter translocation and new protein synthesis [27]. Studies performed in X.oocytes also suggest such an effect, although more investigations are needed to identify the exact mechanisms whereby MET exerts this effect. Recently, the beneficial action of MET on dexamethasone-induced insulin resistance has also been attributed to an effect on glucose transporter intrinsic activity [63]. Thus, while a direct drug action on GLUT translocation is currently questionable, it appears that an improvement of transporter intrinsic activity accounts largely, if not entirely, for much of the increase in glucose transport found after MET treatment. Some studies suggest that the defects in glucose transport in diabetic rats could be divided into two parts: the reduction in basal transport would be due to lower intrinsic activity, whereas limited transporter translocation might account for impaired insulin-induced responses [71]. Such defects are found in insulin-resistant states such as those induced by sucrose [72], dextrose [73] or high fat diet [74], or in mild hyperglycaemia following partial pancreatectomy [75]. This hypothesis might apply for MET, as seen in fructose-fed rats in which chronic treatment with MET normalised skeletal muscle glucose transport mainly in the basal state (Fig. 3). The same mechanism, i.e. improved intrinsic activity, has been claimed to explain increases in adipocyte glucose transport in vitro by myricetin, an effect which was independent of the tyrosine kinase activity of the insulin receptor as well as of GLUT-4 translocation [76].

A very recent investigation revealed that, while MET had no effect per se, it increased the Vmax of glucose transport in the presence of insulin in native oocytes, and reduced Km when oocytes were transfected to express GLUT-4 [77].

Taken together, the available data on effects induced by therapeutic MET levels on glucose transport show that the action of this compound is to boost reactions prestimulated by either insulin or glucose itself. In view of the different subtypes of GLUTs and the different mechanisms involved in both reactions, it is suggested that MET preferentially acts on the capacity of transporters inserted and activated within the cell membrane.

Glucose metabolism A large majority of reports has shown that the major effect of MET on glucose metabolism relates to the non-oxidative pathway, and more particularly to the formation of glycogen. Direct measurements of glycogen levels in biopsies have shown that MET increased glycogen levels in various insulin-resistant tissues in vitro [27, 78] or in vivo [79, 80]. MET has been suggested to increase glycogen cycling with possible net accumulation [80, 81]. Recently, MET was shown to increase the synthesis of glycogen impaired by the addition of glucosamine in cultured myotubes from NIDDM patients [78]. The data from red blood cells show that at least part of this
action was attributable to increased glucose entry into cells. However, in insulin-sensitive tissues, additional regulation of the enzyme (glycogen synthase) by the hormone is involved. The effect of MET on the activation of glycogen synthase is also observed in the absence of glucose when insulin is present [54]. Several studies have shown that MET increases the active form of glycogen synthase [54, 80, 82]. As seen in Figure 4, MET and insulin increased the active form of glycogen synthase at doses at which the drug had no effect by itself. In no case was there a change in total enzyme activity [54]. Therefore, by mechanisms which will be discussed further in this review, MET finally improves a key determinant of insulin resistance, i.e. the defective activity of glycogen synthase.

**Metformin and insulin receptors**

As noted above, MET can exert beneficial effects on some metabolic pathways of glucose, such as gluconeogenesis in the absence of insulin, but usually at relatively high concentrations which are only transient. With remarkable exceptions, studies on glucose transport and metabolism in peripheral tissues have shown that MET at therapeutic drug concentrations requires the presence of insulin, albeit at extremely low doses. This point has been discussed in some detail elsewhere [4]. Recently, our knowledge about the effects of MET on insulin receptors has considerably progressed, in particular through the use of an experimental model well-adapted to investigations at the cell level: the Xenopus oocyte [83]. These cells can easily be manipulated and are not only responsive to IGF-1 but also to the metabolic and anabolic actions of insulin [84, 85].

**Insulin receptor binding** – Before investigations could be conducted on insulin receptor signaling, it was necessary to perform measurements on insulin binding in many experimental and clinical studies about MET. In particular, there was considerable controversy as to whether MET would increase the number of low-affinity receptors in erythrocytes [86]. On the whole, this topic gave controversial data, most of which was negative. Although increases in insulin binding were sometimes seen, analysis performed to relate the changes in binding with the *in vivo* efficacy of the drug failed to reveal such a causal relationship [87-89]. Thus, MET itself was usually considered to have little or no direct effect on insulin binding, and the positive data were due rather to indirect drug effects via general improvement of the diabetic state or to peculiar experimental situations. Over the years, MET was therefore claimed to act on postreceptor steps (a somewhat misleading term mainly signifying postbinding).

**Insulin receptor phosphorylation** – Evidence for an effect of MET on insulin receptor signaling emerged first indirectly when its various effects were compared with the known functioning of the insulin signaling cascade. Indeed, many reports on the biological effects of MET have shown that, with the notable exception of amino-acid transport, the drug increased all other...
biological effects stimulated by the hormone (glucose transport, glycogen synthesis, lipid metabolism, oocyte maturation). Since individual functions are considered to be governed by relatively separate biochemical pathways after docking of specific proteins with phosphorylated IRS-1, it is likely that MET acts behind the binding step, but upstream of the IRS-1 docking process. In other terms, in the absence of a significant effect on hormone binding, the most plausible target appeared to be the insulin receptor itself, in particular the transmembrane and intracellular parts where autophosphorylation and tyrosine phosphorylation take place [90].

Because of the usual controversial findings inherent to pharmacology (probably attributable to the technical characteristics of protocols), it has long been debated whether MET acts on insulin receptor phosphorylation and tyrosine kinase activity. Indirect observations suggested that MET might well be found to increase these parameters and thereby the biological effects of insulin. Indeed, MET has repeatedly been found to enhance the effects of insulin selectively, but not of other agonists, on various biological functions. Thus, the production of albumin in hepatocytes was increased when insulin, IGF-I or EGF was the agonist, but not when thyroxine or dexamethasone was used [91]. In HepG2 cultures, MET modified the response of PAI-1 secretion to insulin but not to IL-1 or phorbol ester [34]. In the X.oocyte, MET increased both the velocity and rate of maturation for insulin, but not for progesterone [92] or MGBG (methylglyoxal-bisguanylhydrasone) [93]. These data are suggestive of a MET action on tyrosine kinase-utilising reactions. Recent measurements showed that MET increased insulin-receptor tyrosine phosphorylation and kinase activity in human diabetics [94] as well as in obese normoglycaemic patients [95], which suggests that this effect was drug-induced and not due to an indirect effect through a reduction in hyperglycaemia. In animals, acute oral administration of MET resulted in a rapid increase in insulin receptor kinase activity [53]. In chronically treated diabetic rats, MET increased skeletal muscle tyrosine kinase activity, even to supranormal levels, which correlated with glycogen formation [81]. In vitro, MET increased the autophosphorylation of the β-chain of the insulin receptor in cultured HepG2 cells rendered insulin-resistant by a change in their membrane cholesterol content and a restoration of their sensitivity to insulin [38, 96]. The use of X.oocytes as experimental tools has allowed investigations to focus on insulin receptor signaling since these cells are an elegant tool for studying cellular processes. Due to their size (spheres about 1 mm in diameter), they can be manipulated easily to inject or sample material through the use of microsyringes. Thereby, data can be collected from individual cells. Many publications have confirmed their usefulness as tools for experimental pharmacology. Studies using oocytes have provided both direct and indirect demonstrations that MET acts on insulin receptor phosphorylation and kinase activity. Thus, initial approaches showed that MET not only increased and accelerated the maturation of these cells in the presence of insulin, but also doubled the phosphorylation of p45annexin [85]. Reevaluation of this effect by other groups confirmed the capacity of MET to potentiate insulin-induced oocyte maturation, but only in the presence of insulin [93, 97]. The increase in tyrosine kinase by MET was not only found in intact whole cells but also in so-called cortical preparations in which the cell interior is absent [97]. Very interestingly, MET also directly stimulated the tyrosine kinase activity of human intracellular β chain in vitro in the absence of insulin [98], thus showing that there is some potential for MET to produce direct insulin-like effects, though much weaker than in the presence of insulin. Once again, selectivity was observed since MET stimulated the activity of three tyrosine kinases (intracellular portions of human insulin or EGF receptors and pp60src) but not of serine/threonine kinase-dependent PKA [98]. The comparison of MET effects with those of structurally close biguanides showed that buformin, which is known to be a stronger-acting drug, induced more intense effects, while mono-methylbiguanide, which is a very weak hypoglycaemic agent, was inactive [98]. The parallel behaviour between the effects of these drugs on insulin receptor tyrosine kinase activity and their in vivo glucose-lowering efficacy also argues in favour of this mechanism as a key determinant in their antidiabetic action. The recent discovery of a particular form of IRS in X.oocytes, and the selectivity and similarity of its effects with those of MET on maturation, also argues for MET action through the insulin signaling pathway [99].

In addition to these proofs for stimulation, indirect observations indicate that MET acted through increased insulin receptor signaling/phosphorylation. Not unexpectedly, cAMP levels after insulin were further reduced upon addition of MET [100]. Moreover, addition of tyrophostin B46, an agent interfering with receptor tyrosine phosphorylation, abolished the effect of combined MET and insulin [100]. It is also noteworthy that MET showed no effect when added after insulin. Glucose transporter intrinsic activity can be inhibited by parathyroid hormone, and in fact this compound eliminated the effects of MET [77]. Interestingly, these mechanisms might account for the enhancement in glucose transporter intrinsic activity described previously since insulin, by dephosphorylating GLUT-4, increases intrinsic activity [101], and an independent decrease in PKA also exerts this effect [101, 102].

In cultured vascular smooth muscle cells, basal tyrosine kinase activity was also increased by MET, indicating that the compound might exert this effect in
most cell types [103]. Conversely, MET had no effect on tyrosine kinase activity in adipocytes in several studies [11, 70, 104, 105]. Interestingly, MET also had no effect on glucose transport at therapeutic concentrations in these studies. Alternatively, adipocytes, which are in many respects different from other cells, may not respond to MET.

Another possibility, poorly explored as yet, is the effect of the drug on phosphatases. An inhibitor effect has been reported in treated Zucker rats [106], but this needs to be confirmed since very recent in vitro measurements in oocytes failed to provide confirmation (D. Detaille, personal communication.). Clearly, more work is needed to elucidate this point.

The relevance of these drug effects for the therapy of patients is obvious if we consider the cardinal role played by defects in tyrosine kinase activity in insulin resistance and NIDDM [107-109].

**Signaling** – Some studies have investigated the effects of MET on the signaling steps behind the insulin receptor. On the basis of evidence given above, elevated rates of activity might be expected for some proteins involved in the cascade. Indeed, MET treatment of ageing rats, which are known to be insulin-resistant, led to an increase in IRS-1 phosphorylation and its association with PI3 kinase in skeletal muscle [110]. In the liver of rats treated acutely with oral MET and given insulin injection in the portal vein, an immediate effect was seen [53], which took the form of an increase in hepatic IRS-2 (but not IRS-1) phosphorylation and PI3 kinase activity. IRS-2 is the main effector of insulin hepatic actions [111].

At steps further downstream, MET was found to increase the amount of IP3 produced in oocyte membranes. This effect was not mediated by a G-protein but was related to tyrosine kinase enhancement, which suggests that MET and insulin activated phospholipase C. In the absence of insulin, MET induced a similar effect, but to a much lower extent not sufficient to activate the cell maturation process by itself [97]. Stimulation of subtypes of PLC by insulin [112] and MET might represent another possibility for a cooperative action of both substances, thereby increasing the biological effect by a synergistic stimulation of common factors. The involvement of second messengers in hormone action, namely phosphatidylinositol-glycans produced by some specific PLC [113] and possibly MET [114], may be another mechanism requiring further exploration. Such a situation would be of interest since the inhibition of cAMP-dependent protein kinase is possibly mediated by an insulin mediator [115], which also increases glucose incorporation into glycogen. These effects have been observed with MET in oocytes [54, 100]. Moreover, PLC has stimulated glucose transporter activity [116].

**METFORMIN AND THE CELL MEMBRANE**

There are several (not mutually exclusive) means by which MET could exert its effects in cell membrane. In theoretical terms, biguanides act as cationic compounds and are thus supposed to bind to negatively charged polar head groups such as phospholipids [117]. This would result in a more positive electrical membrane surface charge, which could influence molecular interactions and glucose transport, as can be seen, for example, with various concentrations of PLC [118]. However, as for several other aspects, MET does not fulfill this expectation since at therapeutic concentrations it binds predominantly to proteins or shows non-specific binding [119], but does not bind to phospholipids [3] (J. Teissie, unpublished results). In direct studies, it has only bound to phospholipids in the mM range. Preferential binding to proteins vs lipid would at least minimise the impact on electrical surface charge, given the respective amount of both classes of products. Moreover, the fact that monomethylbiguanide was inactive, in spite of having the same electrical charge as MET, basically excludes any major causative role of charge modifications in MET membrane effects. An important factor for proper functioning of proteins is their ability to move within the membrane, particularly their lateral mobility. The latter is highly important for vicinal protein-protein or protein-lipid interactions as well as for protein displacement, as in insulin receptor clustering into coated pits or caveolae [113, 120]. Glucose transporters inserted into the membrane must open/close their channels and are also subjected to transmembrane interactions between their exo- and endofacial ends. Additionally, recruited transporters penetrate the plasma membrane by exocytotic membrane fusion and are later endocytosed again [121]. These mechanistic processes are clearly dependent on adequate membrane fluidity. Membranes are commonly viewed as a “fluid mosaic”, with proteins embedded in lipids. Membrane fluidity is a bulk property which changes the conformation of proteins. As a result, their activity can be modulated depending on the accessibility of their reactive sites. According to the nature and duration of the disturbance, as well as the initial state of the membrane, different responses can then be obtained [122].

Many reports have described the dependence of protein functions, such as transport or enzyme activity, on bulk membrane fluidity [123, 124]. Obviously, this topic is very complex due to lipid composition, concentrations of individual lipids and the techniques used [125, 126]. Nevertheless, it is clear that there is an optimal fluidity range and that reduced fluidity is generally linked to impaired transport. For example, moderate increases in fluidity increase glucose transport [127, 128]. In flat cells, basal glucose transport is
not fully active and can be increased further by augmenting fluidity [129], whereas insulin-stimulated glucose transport is decreased when fluidity diminishes [123]. Small changes in fluidity are related to fine changes in glucose transporter unmasking [130]. Concerning insulin receptors, it was found that lower fluidity also reduced the level of messengers activating PDH [131]. Other data have further demonstrated a link between fluidity and insulin receptor microaggregation [132]. Conversely, supranormal fluidity can also impair transport [133, 134].

Glucose or insulin, as well as cholesterol (a major determinant of membrane fluidity), is increased in diabetes. Insulin appears to influence membrane physical state by various possible mechanisms [135] and has generally been found to decrease fluidity in various cell types [134, 136-139]. The reasons for controversial findings with insulin, which have been considered [139, 140], also depend in part on hormone concentrations [133]. Glucose, on the other hand, has been poorly investigated, and the data appear to be equivocal [141-144]. In diabetes, membrane fluidity was found to be increased in leucocytes, while several studies have reported reduced fluidity in platelets and red blood cells from NIDDM patients [145-148]. Once again, modifications may depend on the severity of the disease and on lipid compositional changes [149]. Taken together, these data show a close relationship between membrane physical state and the function of both the insulin receptor and the glucose transporter. Changes in fluidity are therefore a likely mechanism whereby a drug such as MET could modify the functioning of these proteins in diabetes.

**Metformin is a membrane effector**

The concept of biguanides acting on membrane is not new [150]. However, in contrast to other biguanides with longer side chains, MET has low affinity for hydrophobic regions, and many of its effects have proved to be different from those of larger molecules of this class. Since the publication of this last work, many data have accumulated, and a series of specific investigations has been undertaken to clarify this point.

**Biological evidence** – A cell membrane is obviously requisite to obtaining an effect on insulin receptor phosphorylation since several negative reports exist relative to the use of purified receptors. Partially purified receptors seem to induce better drug activity, and the increase in tyrosine kinase activity induced by MET is much weaker with the isolated β chain than with an intact oocyte [96, 98]. The best evidence concerns the observation that a cortical oocyte preparation (i.e. cells emptied and left with the membrane cortex) reacts adequately to the potentiation of insulin receptor phosphorylation by MET, which implies that cell internal components were not required. As will be seen, intracellular penetration of MET is not requisite to induce biological effects in the oocyte. However, when MET was added directly to the oocyte interior by microinjection, an effect was also obtained, but which, contrary to expectations, was weaker for the activation of glycogen synthase [54] and produced a twofold delay for oocyte maturation [93].

Other evidence is as follows: MET seems to act preferentially on receptors linked to tyrosine kinases [91, 98], which is an indirect support for a step located somewhere in the membrane. It affects the intrinsic activity of membrane-inserted glucose transporters and can also interfere with the transport of circulating substances such as glucose and alanine [46, 154]. It corrects for the glycation occurring on the surface of red blood cell ghosts incubated in high glucose [119]. In vascular studies, MET was found to protect against small vessel permeability induced either by diabetes or ischaemia [152, 153], as well as markedly reduce leucocyte adhesion to capillary walls [154]. This points to modifications of the processes occurring on the cell membrane surface (cell-cell interactions). Finally, MET remarkably protects erythrocytes from hypo- or hyperosmotic shock in vitro (A.Duch and N.Wiernsperger, unpublished results).

**Action site: membrane vs cytosol** – As stated elsewhere, drugs with a pKa as high as 11.5 for MET cannot significantly cross membranes if there is no specific carrier. In vitro, MET penetrates cells at extremely low rates (below 1 %) over a period of several hours, which has been found to occur in various cell types as different as hepatocytes, fibroblasts, astrocytes, erythrocytes or oocytes [3]. In vivo, the situation might be somewhat different since a good part of the drug will bind electrovalently with many substances. In fact, the concentration found in rat hepatocytes after oral treatment was above plasma levels and situated mainly in the cytosol [155]. However, this may have been largely due to the fractionation technique used. Our recent washout studies in oocytes revealed that the ionic interactions of MET with the membrane were weak (D. Detaillé, personal communication). Moreover previous studies on MET distribution suggested that in vivo the drug might bind to lipidic structures (chylomicrons, VLDLs) before entering the liver, and could thus be internalised by hepatocytes in a bound form [8]. A search for a possible transport mechanism for MET across membranes revealed that the arginine/lysine transporter system y + might facilitate drug uptake by oocytes [156]. However, there is no proof that the intracellular drug is linked to activity since binding to mitochondria (a frequently claimed mode of action of biguanides) was weak [155] and intracellular MET was found to have no effect on cell respiration when internalised (X. Leverve, personal communication). Moreover, MET is known to have no direct effect on soluble enzymes [48, 150]. We took advantage of the oocyte model to measure both the
intracellular appearance of MET and biological effects such as activation of glycogen synthase. Table II shows that, at the time when the insulin-potentiating effect of MET was fully present, cytosolic drug concentrations were negligible. The much weaker effect obtained when MET was directly microinjected provides further evidence that some sites within the membrane could be the target of the drug. These data provide unequivocal proof that the increase in insulin signaling and functions is completely achievable if the drug is on, or in, the cell membrane. This does not completely exclude the possibility that the intracellular drug might affect other functions, but it is now clear that membrane-located MET is sufficient to generate its full effects on insulin-mediated glucose transport and metabolism.

**Physical evidence** – Through the use of red blood cell ghosts, studies investigating MET binding to erythrocyte membrane have shown that it was non-specific but still preferential for protein bands 4.2 to 4.5 [119]. Interestingly, these bands contain 4.2 (pallidin), which is a protein linked in a mesh to skeletal proteins such as ankyrin or spectrin to ensure membrane integrity [157]. Importantly, these protein bands contain the glucose transporter of the red blood cell (band 4.5) [158]. Thus, the drug might modify cell membrane dynamics, thereby affecting protein configuration, interactions and functioning.

**Membrane fluidity** – Several investigations have been carried out concerning membrane fluidity changes with MET. The effect in systems using black films alone was weak and only seen in the mM range. Moreover, in the presence of albumin, thus in closer correspondence to physiological situations, no further binding to phospholipids and no effect on fluidity were detected, which suggests that MET, at therapeutic concentrations, might not affect membrane lipids. In intact CHO cells, a small fluidising effect was found, which was enhanced in the presence of insulin (J. Teissie, unpublished results). Direct incubation of intact red blood cells from healthy humans with MET showed a fluidising effect, which was confirmed by similar measurements after chronic treatment of NIDDM patients [146]. Measurements with different fluorescent probes suggested that MET influenced membrane lipids, but not those located in deeper layers of the cell membrane (P. Drouin and M. Donner, personal communication).

In a series of specific studies using electron paramagnetic resonance spectroscopy in human red blood cell ghosts, MET decreased the order parameter (i.e., increased the fluidity) of cell membranes in its therapeutic range (0.5-5 \( \mu \)M), whereas higher doses tended to be less active [142]. Moreover, the effect here was more pronounced on the more polar interface of the membrane than in the hydrophobic core. Insulin as well as glucose rigidified these membranes. The coinubcation of ghosts with glucose and MET normalised membrane fluidity, whereas addition of insulin did not modify this reaction. Very high MET concentrations led to an overshoot in fluidity. These data were interpreted as showing a drug effect on proteins at low concentrations and on membrane phospholipids at high supratherapeutic doses.

**SH groups** – SH groups are of utmost importance for the functioning of glucose transporters [159-162] as well as for many effects of insulin. Concomitantly with a loss of fluidity, the thiol status of the membranes, as determined by specific markers such as mono-bromobimane fluorescence, decreased by 40% in 20 mM glucose. The non-enzymatic glycation induced by incubation in hyperglycaemic medium led to a rigidification of the environment of membrane thiols. Coinubcation with either insulin or MET (1-5 \( \mu \)M) counteracted the deleterious effects of high glucose, and both substances were partially synergistic [119]. MET did not interfere with glucose binding to membranes, and the ESR spectrum indicated that there were changes in the configuration of membrane proteins. Once again, low drug concentrations were more efficient than high ones. There was a nice similarity between the improvement in membrane fluidity and thiol reactivity in the presence of MET. Thus, the drug acted on the configuration of glycated proteins, as evidenced by SH group exposure, and this effect was linked to higher membrane fluidity.

Thus, these studies show that there is a convincing correspondence between drug binding to membrane sites comprising glucose transporter and cytoskeletal proteins, membrane fluidity and integrity of the conformation of proteins such as glucose transporters. The notion that MET might modify the conformation of proteins through changes in the physical state of cell membranes also fits with the observations reported previously on insulin receptor phosphorylation, glucose transporter intrinsic activity and changes in \( V_{\text{max}}/K_m \).
LINKS BETWEEN BIOLOGICAL AND PHYSICAL PARAMETERS

MET acts mainly, if not exclusively, at the level of the cell membrane. As described above, it clearly affects membrane fluidity and the lateral mobility of proteins. In normal cells, this effect is minor and may account for the usual absence of direct manifest drug effects on glucose uptake/metabolism at relevant concentrations in a normal milieu. However, it corrects abnormal fluidity in a diabetic environment. Cardinal processes, such as membrane insertion, transmembrane dynamics and unmasking of glucose transporters, are partly governed by the physical properties of the plasma membrane, notably, but also not exclusively, fluidity changes. Normalisation of membrane fluidity towards the optimal physiological range can be expected to unmask transporters, increase their intrinsic activity and favour membrane fusion of transporting vesicles. This would explain the ability of MET to promote the mass action effect of glucose (glucose-mediated glucose transport) even in the absence of insulin. According to this hypothesis, MET would prepare the glucose transporter for maximal efficiency upon subsequent stimulation. It would account for the modifications seen in the kinetic parameters of the glucose transporter without direct action on translocation.

Although the insulin receptor exhibits less lateral mobility than most other proteins, this property is nevertheless needed for receptor collision and transphosphorylation. Changes in receptor configuration are another requirement for adequate phosphorylation and signal propagation, for which a relatively fluid environment is required. It is an old observation that insulin addition to cells induces the formation of membrane ruffles which contain insulin receptors and phosphorytrosines [163]. Clustering of insulin receptors also implies that they move towards and concentrate within specialised structures such as coated pits or, as discovered more recently, caveolae. These are apparently the sites where signaling takes place, since caveolin can phosphorylate IRS-1 [164], which is known to play an important role in insulin effects on glucose transport [166]. Annexin II is another component of caveolae-rich domains [167]. This protein has been shown to be phosphorylated by MET and is indeed involved in insulin receptor processing [168]. Moreover, it is noteworthy that annexins, which act as calcium-binding proteins, are located at the inner site of the plasma membrane [169] and are able to modify membrane properties such as fluidity [170]. pp60src (which can be phosphorylated by MET) belongs to a family of proteins elegantly termed architectins and is also implicated in plasma-cytoskeletal configuration changes [122]. Finally, it should be noted that the IP3-sensitive calcium channel, which is influenced by MET [52], is also found in caveolae [171].

These many aspects show a remarkable similarity with what is observed throughout the numerous data on MET pharmacology. They are certainly at least sufficient to build up a new hypothesis according to which this drug, acting at the membrane level and modifying physical characteristics, influences the configuration and/or interactions of proteins, including those typically involved in glucose transport and its control by insulin. Convincing evidence comes from the comparison between dose-effect curves of MET on tyrosine kinase activity of the insulin receptor, activation of glycogen synthase and membrane fluidity (Fig. 5). It clearly appears that there is an ideal dose range which corresponds to the therapeutic drug level, where all three parameters are modified in a favourable direction by MET. Higher concentrations in vitro reveal a progressive loss of action on the biological parameters, which is paralleled by an inversion in the drug effect on fluidity. In fact, such reversals of biological effects at higher drug concentrations are frequently observed [28, 60, 96, 172] and fit with the observations on cellular mechanisms.

Certainly, not all substances fluidising membranes also reduce hyperglycaemia or insulin resistance. This implies that MET acts on specific microdomains [122] such as the lipid/protein arrangement immediately surrounding the insulin receptor. Alternatively, this effect might only partially explain the cellular effects of MET. It should also be pointed out that these effects in no case exclude additional effects mediated by the intracellular drug.

HYPOTHESIS

The data are compatible with a hypothesis according to which therapeutic concentrations of MET act at the level of the cell membrane and modify its physical state, so that at least microdomains linked to the insulin receptor and glucose transporters become involved. In normal, non-insulin-resistant cells, this effect is either absent or too weak to induce a measurable biological result. However, when cells are insulin-resistant, MET corrects defects in insulin signalling by improving receptor autophosphorylation and tyrosine kinase activity. Due to improved signalling, glucose transport and, more importantly, glycogen synthesis tend to normalise. Thus, MET corrects two main defects underlying insulin resistance in pre-diabetes and NIDDM. When hyperglycaemia is also present, MET improves the intrinsic activity of membrane-inserted glucose transporters, an effect that can occur without insulin. In both cases, data favour protein configuration changes, probably allowed by the correction of membrane fluidity impaired by high glucose or insulin. Although proof is needed, actual findings and recent fundamental discoveries suggest...
that caveolae could be an ideal target accounting for most, if not all, of these effects.

It seems reasonable that such MET-induced modifications in cell membranes are a common denominator in the drug’s many actions in various organs. Differences in biochemical outcome occur according to the respective characteristics and actual state of the different cell types.

**A REMAINING ENIGMA**

However, there is still an unanswered question: what is the nature of this peculiar relationship between MET and insulin? Certainly, there is a multitude of data showing an increased effect of insulin in the presence of MET, which argues in favour of a potentiation of hormone action. Nevertheless, clearcut data also exist which show that extremely low insulin concentrations can cause large biological effects in the presence of MET. These data suggest that the synergism of both substances is at least bidirectional. One possibility is that small, but identical, effects induced by each substance add up to reach a critical threshold, e.g. the production of IP3 in oocytes. It seems unlikely, however, that insulin concentrations as low as traces could induce a measurable effect. MET and insulin could act on complementary processes, e.g. the hormone could unmask glucose transporters, whereas MET could stimulate its transport capacity. Alternatively, the complex formed by MET and insulin, which appears to be present at a rate of about 2 % of the drug in plasma [4] could be the active compound, with insulin eventually behaving as a vector. This would explain the fact that MET requires some hormone and expresses its clinical efficacy usually after some days of treatment. Interestingly, it was shown by Gould and Chaudry that insulin at low concentrations had a permissive effect for its own action at slightly higher doses [21]. These authors had already postulated that insulin acts by modifying the physical state of the cell plasma membrane, and other authors have since proposed that the quite significant non-receptor binding of insulin to membranes might well have definite effects by this route [122, 135]. In this context, the case of red blood cell glucose transport deserves special attention since it exemplifies how preincubation with low insulin of cells otherwise unresponsive to the hormone [173, 174] can further increase the effect of MET by about 25 %. We may speculate that insulin, by slightly modifying membrane surface characteristics, could direct MET towards specific microdomains, such as the hormone receptor environment, or change drug partitioning in the membrane itself. This is a fascinating enigma since it suggests that some still unidentified sites could be potent therapeutic targets even in cases of severe insulin deficiency.

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