Mitochondrial metabolism and type-2 diabetes: a specific target of metformin

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SUMMARY
Several links relate mitochondrial metabolism and type 2 diabetes or chronic hyperglycaemia. Among them, ATP synthesis by oxidative phosphorylation and cellular energy metabolism (ATP/ADP ratio), redox status and reactive oxygen species (ROS) production, membrane potential and substrate transport across the mitochondrial membrane are involved at various steps of the very complex network of glucose metabolism. Recently, the following findings (1) mitochondrial ROS production is central in the signalling pathway of harmful effects of hyperglycaemia, (2) AMPK activation is a major regulator of both glucose and lipid metabolism connected with cellular energy status, (3) hyperglycaemia by inhibiting glucose-6-phosphate dehydrogenase (G6PDH) by a cAMP mechanism plays a crucial role in NADPH/NADP ratio and thus in the pro-oxidant/anti-oxidant cellular status, have deeply changed our view of diabetes and related complications. It has been reported that metformin has many different cellular effects according to the experimental models and/or conditions. However, recent important findings may explain its unique efficacy in the treatment of hyperglycaemia- or insulin-resistance related complications. Metformin is a mild inhibitor of respiratory chain complex 1; it activates AMPK in several models, apparently independently of changes in the AMP-to-ATP ratio; it activates G6PDH in a model of high-fat related insulin resistance; and it has antioxidant properties by a mechanism(s), which is (are) not completely elucidated as yet. Although it is clear that metformin has non-mitochondrial effects, since it affects erythrocyte metabolism, the mitochondrial effects of metformin are probably crucial in explaining the various properties of this drug.

Key-words: Metformin · Mitochondria · Type 2 diabetes · Mechanismal action · Glucose metabolism · Diabetic complications.


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The tremendous worldwide increase of patients suffering from type 2 diabetes is probably one of the leading health concerns for the future [1]. This is due to (i) the rapid extension of the disease, (ii) the rate of occurrence and the severity of the induced complications, related to both micro- and macro-angiopathy, and of course (iii) the cost of its care, which can be estimated at 100 billions US dollars per year, i.e. 15% of total US health care annual costs [2-4]. Although type 2 diabetes is a disease classically known as “adult-onset diabetes” occurring in First World well-developed countries, it is actually now a disease affecting young people living in the less developed countries of the Third World [4, 5]. It is well established that the disease is related to both a specific genetic background and a particular life style based on high caloric intake and low exercise. Moreover, the sequence of pathophysiological events is also characterised by the succession of (i) obesity-related insulin resistance, (ii) glucose intolerance, (iii) type 2 diabetes, (iv) decrease in the capacity of insulin secretion by pancreatic β-cells, leading finally to (v) an “end of life type 1 diabetes”. However, the actual mechanism responsible for the deleterious complications of the disease is still the matter of active research since it is not fully elucidated.

As long been suspected by the diabetologists, several lines of evidence indicate clearly that hyperglycaemia is not only a marker of the disease and its severity, but also a causal event responsible per se for most of the deleterious consequences of this disease [6]. Hyperglycaemia is advocated as a main underlying metabolic abnormality in type-2 diabetes [6] and in inflammation-related insulin resistance in intensive care patients [7]. Regarding the deleterious effect of hyperglycaemia, Brownlee proposed a unifying hypothesis based on superoxide overproduction from the mitochondrial electron transport chain as a consequence of hyperglycaemia-related increased glycolysis [8]. Hence, if reducing hyperglycaemia as early and as deeply as possible remains the cornerstone in the treatment of diabetic patients [9], decreasing the reactive oxygen species (ROS)-related glucose toxicity at cellular level may represent an additional attractive perspective. Mitochondria appear to play a crucial role not only in some particular diabetes [10], but also in the pathogenesis of the deleterious events related to any hyperglycaemic state, and superoxide overproduction at the level of the respiratory chain represents probably an important target in the treatment of diabetes.

The treatment of type 2 diabetes is mainly based on two different additive approaches: a change in life style (diet and exercise), and a pharmacologically induced decrease in blood glucose [9, 11]. Among the different drugs used for the treatment of type 2 diabetes, metformin is widely used [12-14] since it lowers glucose by increasing muscle glucose uptake [15] and decreasing hepatic glucose production [15-17]. However, the underlying cellular mechanism responsible for the antihyperglycaemic effect is still poorly understood. Interestingly, as reported in the large UKPDS survey, it seems that the beneficial effect of metformin is attributed not only to a decrease in blood glucose, but also to an action of the drug beyond its glucose-lowering effect [6]. Antioxidant effects of metformin have been reported in various models, including humans [18-24]. The purpose of this review article is to examine the relationship between metformin, mitochondrial metabolism and carbohydrate metabolism.

Glucose metabolism and cellular energy status

In the human body considered as a whole, a decrease in hyperglycaemia can be obtained by lowering glucose entry to the system (i.e. glucose absorption and/or gluconeogenesis or glycogenolysis), by increasing glucose removal (i.e. glucose oxidation, metabolism and/or glycogen storage), or by both. Several studies have shown that metformin affects intestinal glucose absorption; however, this effect cannot explain its hypoglycaemic action, and the major effect of the drug is on glucose metabolism [25]. Metformin stimulates insulin-induced glucose uptake by skeletal muscle and adipocytes in both diabetic individuals and animal models. This increase is more pronounced in diabetic than in non-diabetic animals, suggesting an enhanced action of the drug in the hyperglycaemic state. Metformin also affects the insulin-dependent part of the pathway of glucose oxidation. Metformin acts potentially via the insulin receptor and the glucose transporters: metformin increases insulin binding and a direct effect on the glucose-transport system has been demonstrated. According to the model, this effect is either additive to insulin, suggesting an insulin-dependent action, or independent from it. These results led to the proposal that the antihyperglycaemic effect of metformin is located at the level of skeletal muscle by increasing glucose transport across the cell membrane.

Nevertheless, several studies suggest that metformin is responsible for a decrease in the glucose release from liver both in vitro and in vivo [16, 17]. Glucose synthesis from 3-carbon precursors (lactate, alanine, glycerol) is an ATP-consuming pathway, which is very sensitive to any alteration in ATP metabolism. In isolated liver cells we have reported that the decrease in gluconeogenesis was due to an increase in the flux through pyruvate kinase [16]. In these experimental conditions of isolated liver cells, we found that metformin was responsible for a mild but significant decrease in ATP/ADP ratio in both cytosolic and mitochondrial spaces. Since pyruvate kinase is exquisitely regulated by an allosteric regulation not only via a c-AMP dependent phosphorylation but also by several powerful effectors including ADP and ATP [26], it was proposed that metformin is responsible for an increased intracellular cycling at the level of pyruvate/phosphoenolpyruvate related to a change in ATP metabolism and leading to a decreased rate through gluconeogenesis associ-
ated to some energy wasting [16, 27]. Metformin, which is an antihyperglycaemic agent, has no marked hypoglycaemic effects, which could be explained by the opposite effects of metformin and glucagon at this level [16]. Indeed, hypoglycaemia results in a rise in glucagon, which lowers the effect of metformin on pyruvate kinase and thus leads to increased liver glucose production.

Considering the glucose pathway as a whole, i.e. glycolysis, pyruvate oxidation, gluconeogenesis, glycogen metabolism, there is a tight relationship between cellular glucose metabolism and energetic status. Indeed, ATP and phosphate potential (ATP/ADP):P) are involved in many of the key steps of glucose metabolism, this connection being responsible for a tight link between mitochondrial oxidative phosphorylation regulation and glucose homeostasis.

In recent years, adenosine 5'-monophosphate-activated protein kinase (AMPK) has appeared as a "metabolic master switch" in cellular energetic homeostasis [28]. Indeed, by phosphorylating key target proteins, it permits to control the flux through several major metabolic pathways such as hepatic ketogenesis, cholesterol synthesis, lipogenesis, triglyceride synthesis, adipocyte lipolysis, and skeletal muscle fatty acid oxidation. AMPK activation mediates the stimulation of glucose uptake induced by muscle contraction and it stimulates fatty acid oxidation and ketogenesis in liver, and fatty acid oxidation and glucose uptake in skeletal muscle [28]. It inhibits cholesterol synthesis, lipogenesis, and triglyceride synthesis in liver. In adipocyte it inhibits lipolysis and lipogenesis. AMPK is activated by muscle contraction resulting in a stimulation of glucose uptake, and in addition it modulates insulin secretion by pancreatic β-cells. Given these metabolic properties, it could be hypothesised that a defect in the fine-tuning of AMPK regulation is involved in the pathogenesis of type-2 diabetes. Indeed, it is already known that exercise, which activates AMPK, is effective in correcting insulin resistance in patients with impaired glucose tolerance and type 2 diabetes. Recently it has been shown that metformin activates AMPK, resulting in an activation of fatty acid oxidation while the expression of lipogenic enzymes is suppressed [29]. Interestingly, the effect is apparently not mediated by a change in AMP-to-ATP ratio, conversely to the effect of rosiglitazone [30, 31]. The effect of metformin on AMPK has been also reported in human studies [32].

Glucose metabolism and ROS production

Although it is long known that hyperglycaemia has several detrimental effects, the understanding of the causal relationship between abnormal high glucose and cell metabolism has only been clarified recently. Based on data obtained in various experimental models, it appears that three pathways have been involved in the pathogenesis of the deleterious effect of hyperglycaemia: (i) glucose-induced activation of protein kinase C (PKC) isoforms, (ii) increased formation of glucose-derived advanced glycation end products, and (iii) increased glucose flux through the aldose reductase pathway. Brownlee proposed an unifying hypothesis based on superoxide overproduction from the mitochondrial electron transport chain as a consequence of hyperglycaemia-related increased glycolysis [8]. It appears that cellular glucose sensing is closely related to reactive oxygen species (ROS) metabolism [8, 33-36]. High extracellular glucose enhances glucose activation and the glycolytic pathway, resulting in an enhanced pyruvate formation. Pyruvate oxidation in the mitochondria is associated with an increase in the mitochondrial membrane potential (ΔΨm). High ΔΨm is responsible for an overproduction of ROS, which in turn inhibits glycolysis by a negative feedback located on the glyceraldehydephosphate dehydrogenase (GAPDH). Hence, the flux of carbon is then rerouted towards the glucosamine pathway, which is responsible for the transcriptional consequence of high extracellular glucose. According to this view, high glucose is thus associated with an enhanced ROS production, this effect being responsible for the harmful consequences of hyperglycaemia. This view represents a major advance in our understanding of the deleterious effects of hyperglycaemia.

Cellular carbohydrate metabolism is related to oxidative status and ROS production via the pentose phosphate pathway (PPP). The main function of this pathway is to produce the reducing equivalent NADPH, compulsory cofactor for several major cellular functions, lipogenesis for instance. NADP/NADPH couple plays a crucial role in the cellular oxidant/antioxidant homeostasis [37]. Indeed, as cofactor of the glutathione reductase, it allows the regeneration of reduced glutathione after it has been oxidised while scavenging hydrogen peroxide (H2O2). Hence, the flux through the PPP is a major way, unique in erythrocyte, for the metabolism of H2O2, indicating that it is deeply involved in the H2O2-related general cell sensing system. By contrast, NADPH is also a unique substrate for the production of ROS by neutrophils at the level of the plasma-membrane-linked NADPH oxidase. Therefore, activation of PPP is expected to increase the production of ROS in neutrophils, reinforcing thus the main function for the killing of bacteria. In summary, it appears that NADPH metabolism via the flux through PPP is involved in both the ROS scavenging pathway and the ROS producing pathway. Recently, it has been shown that high glucose is in fact a powerful inhibitor of the first step of PPP, the glucose-6-phosphate dehydrogenase (G6PDH), by a cAMP-dependent mechanism [38]. Hence, this very important finding represents another crucial link between oxidant/antioxidant status and glucose homeostasis, explaining why abnormally high glucose leads simultaneously to a lower antioxidant defence with an increased ROS production in some cells (e.g. endothelial cells) and to a decrease in ROS production in others, like neutrophils, impairing thus their bactericide function [38-40].
This finding is in good agreement with data reporting an impaired antimicrobial function related to a G6PDH deficiency in humans [40-42].

**Mitochondria and cell death commitment**

Mitochondria are mostly viewed as specialised cellular micro-organelles in charge of energetic supply through ATP synthesis via the oxidative phosphorylation pathway. This function is indeed prominent in living organisms, but due to the specific features of this pathway (i.e. formation of single unpaired electron at the level of respiratory chain complexes 1 and 3 in the presence of molecular oxygen and of a high membrane potential), it is potentially extremely harmful due to the production of hazardous ROS production. Moreover, due to the requirement of a tight adjustment between energy demand and energy supply, since ATP cannot be stored and because of the peril of energy exhaustion, this pathway is in the centre of a complicated cell sensing/signalling system including redox state, membrane potential, phosphate potential and calcium homeostasis. Extended works during the last decade have greatly contributed to emphasise the central role of mitochondria in the regulation of several main cellular functions, including the control of cell death. Although the exact mechanism relating mitochondria and cell death still requires some clarification, it is highly probable that the mitochondrial Permeability Transition Pore (PTP) is involved via the release of cytochrome c [43, 44]. Although the molecular nature of the PTP is still unknown, its modulation by several physiological factors has been widely studied [43, 44]. Among many factors, Ca²⁺ and ROS are very important and ciclosporin A (CsA) is regarded as the specific reference inhibitor [43-46]. Based on several results obtained in various laboratories and experimental models, the following scheme can be proposed: upon a distress signal such as high calcium or overproduction of ROS, a mitochondrial permeability transition occurs, i.e. membrane potential is collapsed, ATP synthesis is impaired, NADH-to-NAD ratio is depressed as well as oxygen consumption. Although the actual mechanism is not completely clear as yet, this phenomenon of permeability transition is linked to the translocation of pro-apoptotic factors, including cytochrome c, which are normally located in the intermembrane space. Cytosolic cytochrome c is then responsible for an activation of the caspase cascade leading to the commitment of apoptosis, probably in conjunction with other proapoptotic factors. In the last years, we have reported that the permeability transition is also modulated by the electron flux through respiratory chain complex 1 [45, 46]. Furthermore, by investigating the effects of an inhibitor of complex 1, rotenone, we found a significant inhibition of the permeability transition, which is associated with a prevention of cell death [47]. Indeed, we showed that rotenone is as potent as ciclosporin A in inhibiting Ca²⁺-induced PTP opening or tert-butyl hydroperoxide (a cellular oxidising agent) induced PTP-related cytochrome c release and cell death.

**Metformin, mitochondria, glucose metabolism, ROS and cell death**

Metformin has undoubtedly non-mitochondrial effects since it affects erythrocyte metabolism, a mitochondrion lacking cell [48-51]. It has been recently evidenced that it affects also mitochondrial metabolism. Several members of the biguanide family are responsible for marked inhibitory effects on the respiratory chain in isolated mitochondria [52-54]. Among these members, phenformin is responsible for a strong inhibitory effect. This compound, which was a potent antidiabetic drug, was withdrawn because of serious side effects (metabolic acidosis with hyperlactatemia). Interestingly, metformin has no effect on isolated mitochondria, probably because of specific physico-chemical properties [52-56]. However, as mentioned above, metformin was found to be responsible for a decrease in cellular ATP or ATP-to-ADP ratio, indicating some effect on the oxidative phosphorylation pathway, although observed at high pharmacological concentrations [16, 57]. Metformin does not affect oxidative phosphorylation in isolated mitochondria, or permeabilised cells, conversely to other members of the biguanide family [52-56]. By investigating this phenomenon, we showed that metformin is indeed a mild inhibitor of the respiratory chain but this effect was found only when intact cells were exposed to the drug [55]. Very interestingly, the mitochondrial effect persisted when mitochondria were isolated after cells, organ (liver), or whole animal (rat), were exposed to metformin in either cell preincubation, liver pre-perfusion or in vivo IP injection [55]. This finding indicated that metformin has probably a mitochondrial target, but this target can be reached only in living cells. Because metformin cannot be metabolized [14], and since this phenomenon was suppressed at low temperature (21°C) and completely insensitive to any inhibitors of signalling pathways [55], we hypothesised that metformin could enter the cell via a plasma membrane-related mechanism. Further support of this view was obtained in a model of *Xenopus laevis* oocytes [56]. In this model, metformin was effective only in intact cells, as in rat liver cells, but not in isolated mitochondria. Interestingly, when metformin was encapsulated into liposomes, its inhibitory effect on mitochondria was present [56]. Therefore, we propose that metformin is indeed a mild inhibitor of respiratory chain, but it needs a plasma membrane related event before it is effective [56]. This view is compatible with data showing that metformin can affect isolated mitochondria but only after 24 h to 60 h, conversely to phenformin which is immediately effective.

Hence, metformin appears to inhibit mitochondrial respiratory chain specifically at the complex 1 level, no other effect being found at any other step of this pathway. This maximal inhibitory effect of metformin on complex 1 is lower than that of the reference inhibitor of complex 1, rotenone (approximately 40% of maximal inhibition with metformin compared with 80% with rotenone). Because met-
formin appears to be a mild inhibitor of complex 1 and in the view of the data presented above, regarding the effect of rotenone on mitochondrial permeability transition, cytochrome c release and ROS-related cell death, we are presently investigating the effects of metformin on these parameters. Preliminary results show that metformin is as potent as ciclosporin A, the reference inhibitor, in preventing cytochrome c release and ROS-related cell death, we are view of the data presented above, regarding the effect of XM Leverve et al. presently investigating the effects of metformin on these (i) the Ca\(^{2+}\)-induced permeability transition, (ii) the ROS-related cytochrome c release and (iii) cell apoptosis commitment (Guigas et al., Detaille et al., unpublished). Because of the close relationship between ROS production, PTP regulation and cell death, we have also investigated the effect of metformin on mitochondria-related ROS production. Preliminary results show that metformin is responsible for a clear decrease in complex 1-linked ROS production, but not in complex 3-linked ROS production (i.e. after antimycin addition). If confirmed, these preliminary results would indicate that metformin does not act as a ROS scavenger, conversely to a recent report [20], but most probably as an inhibitor at the complex 1 level (Batandier et al., unpublished).

Several reports in the literature have investigated the effect of metformin on oxidative stress [18-24]. The overall effect is in good line with our preliminary data indicating that metformin has some antioxidant properties, although the actual mechanism is not completely clear as yet. It is of interest to emphasise the work by Faure et al. on a rat model of insulin resistance in the absence of actual hyperglycaemia and diabetes, the model of high fructose [23]. Indeed, these authors have clearly shown that such model of both insulin resistance and oxidative stress was sensitive to metformin, which improved simultaneously both insulin resistance and antioxidant status. Interestingly enough, this beneficial effect was obtained while this model does not exhibit a chronic increase in blood glucose, indicating that metformin cannot act through its glucose lowering effect. Therefore, this effect is probably related to an antioxidant effect. Such beneficial effect of metformin in the high-fructose model was reported in other works [18, 58]. Fructose was recently found to be an inhibitor of G6PDH [38]. Therefore, the following scheme could be then proposed: (i) inhibition of G6PDH by high fructose leading to (ii) a decrease in the PPP flux and in the related NADPH formation, which (iii) increases ROS production. Because of the lower antioxidant defence, resulting (iv) in an inhibition of glycolysis at the level of GAPDH (Brownlee's proposal) and (v) to a re-orientation of the glycolytic intermediates towards hexosamine pathway, with its deleterious consequence of insulin resistance, even in the absence of hyperglycaemia. One could propose that the beneficial effect of metformin is due to its antioxidant/oxidative stress-protective effect allowing prevention of the deleterious effect of fructose on NADPH/NADP ratio and its related deleterious effects. It is of interest to underline that the beneficial effect of metformin on both insulin resistance and antioxidant status was obtained in this case without hyperglycaemia and therefore any blood glucose lowering mechanism can be excluded. In addition, in a different model of insulin resistance, the high fat regimen, Mitieux et al. have reported that metformin was responsible for an increase in G6PDH activity in the high fat group, but not in controls [59]. This finding is indeed in good line with a beneficial effect of metformin occurring mainly through its antioxidant effect linked to both a decrease in the mitochondrial ROS overproduction and to an increase in PPP-related ROS formation, thanks to the restoration of G6PDH activity.

The desert gerbil _Psammomys obesus_ as a model for metformin-sensitive nutritional type 2 diabetes

From the above-mentioned findings as well as other data in the literature [60-64], the following scenario regarding carbohydrate homeostasis in _Psammomys_ could be proposed. Because of a low rate of glucose 6-phosphatase hydrolysis [64], these animals sustain concomitantly low blood glucose together with high intracellular phosphorylated carbohydrate intermediates, including G6P. Insulin is relatively high, regarding the very low blood glucose [60, 63, 64], allowing these animals to achieve an active lipogenesis [60]. Indeed, despite the low caloric content of the natural food, these animals exhibit a significant lipid storage as illustrated by their name, _Psammomys obesus_, even in their natural biotope [63]. At the same time, the peripheral insulin resistance [60] permits to spare the precious molecules of glucose, which are not present in the food and therefore must be built by gluconeogenesis, for insulin-independent pathways. This state can be viewed as a physiological state of marked insulin-resistance with active lipogenesis and lipid storage, but without systemic hyperglycaemia, even more, with a permanent state of hypoglycaemia. Hence, these animals are protected against the potentially negative effect of insulin resistance by the low blood glucose. When carbohydrates are present in the diet, this delicate equilibrium is impaired and the combination of (i) further insulin secretion together with insulin resistance, (ii) amplification of lipid storage leading to enlarged obesity and (iii) high blood glucose, is responsible for the detrimental effects on \(\beta\)-cells, leading to lower insulin secretion and finally to ketoacidosis. Hence, it is possible to propose that in these animals the low glucose 6-phosphatase activity is the adaptive response to hypocaloric regimen, with very low carbohydrate content [64]. This permits to maintain a high intracellular concentration of phosphorylated sugars, despite the low glucose concentration. In this condition, the potential toxicity of high blood glucose (but normal if compared to Wistar rat) from an exogenous source will be dramatically amplified, leading to the rapid evolution of the diabetic disease, which is well described in these animals when fed with a normo/hypercaloric chow [60]. A comparison between this natural history of diabetes in _Psammomys_ and in type 2 diabetes in man is of interest, it could help in explaining, at least
partly, the high prevalence of type 2 diabetes following changes in the diet, in developing countries, even in non-obese people. Interestingly, metformin is very active in such specific models of nutritional diabetes [65].

References


