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Effect of Metformin on Hepatocyte Insulin Receptor Binding in Normal, Streptozotocin Diabetic and Genetically Obese Diabetic (ob/ob) Mice

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Summary. The effect of metformin on hepatocyte insulin receptor binding was examined in normal, streptozotocin diabetic and genetically obese diabetic (ob/ob) mice. In normal mice, chronic administration of metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$ for 50 weeks) increased the number of low affinity receptors by 148%. During acute studies, metformin increased (30%) the number of low affinity receptors after 24 h. When metformin was withdrawn after treatment for 96 h, the number of low affinity receptors decreased, approaching control values by 48 h. In severely insulin resistant ob/ob mice, the concentrations of high and low affinity receptors were reduced by 60% and 27%, respectively. A high dose of metformin ($240\,mg \cdot kg^{-1} \cdot day^{-1}$ for 4 weeks) increased the concentration of high and low affinity receptors in ob/ob mice by 63% and 86%, respectively. However, the hypoglycaemic response to exogenous insulin was not altered. In streptozotocin-diabetic mice, the number of low affinity receptors was increased by 68% compared with normal mice. Metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$ for 10 weeks) did not significantly alter the number of insulin receptors in streptozotocin-diabetic mice, but the hypoglycaemic response to exogenous insulin was improved by 94%. The results raise the possibility that metformin might affect post-receptor sites of insulin action independently of effects at the receptor level.

Key words: Metformin, insulin receptor binding, hepatocytes, streptozotocin, ob/ob mice.

Introduction

Metformin reduces hyperglycaemia without elevation of plasma insulin in human non-insulin-dependent diabetes [1] and DBM mice [2]. Metformin also increases insulin mediated glucose uptake by diaphragm muscle of alloxan diabetic rats [3]. These observations suggest that metformin potentiates insulin action in diabetic states, inviting the study of a possible influence of metformin on insulin receptor binding. Recently, it has been reported that metformin increased insulin receptor binding in erythrocytes of normal subjects [4] and in IM-9 cultured lymphocytes and MCF-7 cultured breast cells [5]. The present study examines the effect of metformin on hepatocyte insulin receptor binding in two animal models of hyperglycaemia: the hypoinsulinaemic streptozotocin-diabetic mouse and the hyperinsulinaemic ob/ob mouse. The study determines the time and dose characteristics of the effects of metformin on insulin receptor binding.

Materials and Methods

Animals

Male Theiller Original mice (Bantin & Kingman, Hull, UK) and male genetically obese diabetic (ob/ob) mice and homozygous lean (+/+) mice from the Aston colony were used. The origin and characteristics of Aston ob/ob mice have been described elsewhere [6, 7]. Mice were housed in an air-conditioned room at 22 ± 2 °C, and supplied a standard pellet diet (Mouse breeding diet, Heygate & Sons, Northampton, UK) and tap water ad libitum.

Chemicals

Reagents of analytical grade and distilled water were used throughout. The chemicals and their sources were as follows: streptozotocin, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), (N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid)2-((2-hydroxy-1, 1-bis(hydroxymethyl)-ethyl)aminoethanesulphonic acid) (TES), ethyleneglycol-bis-(β-aminoethyl ether)-N, N, N′, N′-tetracetic acid (EGTA), tricine, bacitracin and collagenase (batch C5138) from Sigma (London) Chemicals, Poole, UK; monocomponent porcine insulin (lot S8391272) from Novo Industria, Copenhagen, Den-

mark; Na ^{125}I from Amersham International, Amersham, UK; insulin free bovine serum albumin (fraction V) (batch 318) from Miles Laboratories, Slough, UK; sodium pentobarbitone (Sagatal) from May & Baker, Dagenham, UK; other reagents from British Drug Houses, Poole, UK. Pure metformin hydrochloride (batch 304) was supplied by Rona Laboratories, Hitchin, UK.

Experimental Procedure

Chronic Studies in Normal and Streptozotocin Diabetic Mice: Experiments were begun at 10–15 weeks of age. Streptozotocin (100 mg/kg in citrate buffer, pH 4.8) was administered by IP injection. After 2 weeks, mice were assigned to equi-hyperglycaemic groups (mean plasma glucose concentrations 16.9 and 17.3 mmol/l for control and test groups, respectively). Normal mice were treated with metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$) for 50 weeks, and streptozotocin diabetic mice were treated with the same dose of metformin for 10 weeks. The metformin was administered in the drinking water, and control mice received ordinary drinking water. The dose of metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$) corresponds with the manufacturer's maximum recommended clinical dose on a weight for weight basis. Fluid intake was monitored, and the concentration of the drug was adjusted accordingly, as described elsewhere [8]. The procedure involves daily measurement of drinking fluid intake using a graduated drinking dispenser with a spill-collector. The concentration of the drug in the drinking fluid is adjusted on a daily basis in short-term studies and on a weekly basis in long-term studies to provide the required weight-related dose of the drug.

Acute Studies in Normal Mice: To examine the time dependency of the effect of metformin, groups of 20-week-old normal mice received metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$) in the drinking water for 24, 48, 72 and 96 h. The effect of metformin withdrawal was examined in groups of 20-week-old normal mice after treatment with metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$) for 96 h. Tests were performed 24 and 48 h after the drug was withdrawn.

Studies in ob/ob Mice: The effect of metformin (120 and 240 $mg \cdot kg^{-1} \cdot day^{-1}$ for 4 weeks) administered in the drinking water was examined in 20-week-old ob/ob mice. Untreated ob/ob mice were compared with untreated age-matched homozygous lean (+/+) mice.

Insulin Binding Studies

Blood samples (30 µl) were obtained from the cut tip of the tail of fed mice at 10.00 h on the day of the insulin binding study. Plasma was separated and assayed for glucose [9]. In some studies, plasma insulin [10] and plasma metformin [11] were also measured. Mice were anaesthetised with sodium pentobarbitone (50 mg/kg) by IP injection, and hepatocytes were isolated by a modification of the method of Kahn et al. [12]. The liver was perfused in situ at 6 ml/min with a Ca^{++} free HEPES buffer containing EGTA (1.28 mmol/l) and sodium heparin (160 U/l) for 3–4 min, followed by Ca^{++} free HEPES buffer alone for 3–4 min, followed by HEPES buffer containing Ca^{++} (425 mmol/l) and collagenase (500 mg/l) for 4–5 min, until the liver was approximately twice its original size. The liver was removed, rinsed in HEPES buffer number 4 [13] and disintegrated by gentle manual agitation. The cell suspension was filtered through a 125 µ nylon mesh filter (Sericol, Birmingham, UK) and centrifuged at 10 g for 5 min at room temperature. The cells were washed and centrifuged twice, and filtered through a 60 µ nylon mesh filter. Cell viability, assessed by trypan blue exclusion for 2 min was always > 75%. Hepatocyte number and diameter were determined using a Neubauer haemocytometer (Phillip Harris Biological, Oldmixon, UK) and cell number was adjusted to 2.0×10^6 cells/ml.

Isolated hepatocytes were incubated at 22 °C in HEPES buffer number 4 (pH 7.8) containing 1% bovine serum albumin, 0.5–500 ng/500 µl monocomponent porcine insulin, 800 mg/l bacitracin to reduce insulin degradation [14], and 0.2 ng/500 µl ^{125}I-monoiodinated porcine insulin, in a final volume of 500 µl. The labelled insulin was prepared by the Chloramine T method [15] to a specific activity of 215–230 µCi/µg. All incubations were performed in triplicate in 1.5 ml capacity polyethylene microfuge tubes (PPR15, Beckman Riic, High Wycombe, UK). Incubations were terminated after 120 min by addition of 1.0 ml of ice-cold HEPES buffer and the tubes were centrifuged at 9000 g for 1 min in a Beckman microfuge (type B, Beckman Riic). The pellet was washed once with ice-cold HEPES buffer, and the tip of the tube was excised and counted in a gamma spectrometer. Results were corrected for non-specific binding by subtracting the amount of radioactivity bound in the presence of excess porcine insulin (50 µg/tube) [15]. Non-specific binding was 4.4 ± 0.5% (mean ± SEM) of the total binding to hepatocytes.

Data were analysed using a Scatchard plot [16] to determine the number of receptors. The Scatchard plot for hepatocyte insulin receptor binding is curvilinear. It is not established whether this represents two or more classes of receptors with different but fixed affinities, or one class of receptors exhibiting negatively cooperative site-site interactions [17]. The present data were interpreted by computer-assisted analysis (ICL 1904S, International Computers, London, UK), using a model which assumes two classes of receptors, one with a high affinity and one with a low affinity [18]. The apparent receptor affinity was taken as the concentration of native insulin required to reduce by 50% the specific cell bound fraction of ^{125}I-insulin [19].

Insulin Hypoglycaemia Tests

Insulin hypoglycaemia tests were performed at 10.00 h in fed streptozotocin-diabetic and ob/ob mice 1 week before the insulin binding study. Blood samples (20 µl) were obtained from the cut tip of the tail immediately before, and at 15, 30, 60 and 90 min after an IP injection of monocomponent porcine insulin (0.15 U/kg in streptozotocin-diabetic mice and 100 U/kg in ob/ob mice). Plasma was separated and assayed for glucose [9].

Statistical Analysis

Student's t-test was used for statistical comparisons. Differences were considered to be significant if $p < 0.05$.

Results

Chronic Studies in Normal Mice

Table 1 shows body weights, plasma glucose and insulin concentrations and hepatocyte insulin receptor status in normal mice treated with metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$) for 50 weeks. Metformin did not alter body weight or plasma insulin concentrations significantly, although a small increase in plasma glucose concentrations was observed, as previously reported [8]. The total number of hepatocyte insulin receptors was increased twofold by the metformin treatment and was due to an increase in the number of low affinity receptors. There was no significant effect on the number of high affinity receptors, or the apparent affinity constant.

Acute Studies in Normal Mice

Normal mice treated with metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$) showed an increase in the number of low affinity hepatocyte insulin receptors after 24 h (Table 2). The effect was similar up to 96 h. There was no significant al-

Table 1. Chronic effect of metformin (60 mg·kg^{-1}·day^{-1} for 50 weeks) on hepatocyte insulin receptor status in normal mice

Mice	Body weight (g)	Plasma glucose (mmol/l)	Plasma insulin (ng/ml)	Total receptor number (per cell × 10^5)	High affinity receptor number (per cell × 10^5)	Low affinity receptor number (per cell × 10^5)	Apparent receptor affinity constant (nmol/l)
Control	46.0 ± 0.5	7.4 ± 0.3	2.94 ± 0.59	2.88 ± 0.30	1.25 ± 0.19	1.61 ± 0.40	1.65 ± 0.26
Metformin	49.2 ± 2.3	10.0 ± 0.4[a]	3.34 ± 0.61	5.29 ± 0.41[b]	1.19 ± 0.06	4.00 ± 0.41[b]	2.13 ± 0.27

Results are expressed as mean ± SEM of four mice
[a] $p < 0.01$
[b] $p < 0.02$ compared with control group

Table 2. Acute time-dependent effect of metformin (60 mg·kg^{-1}·day^{-1}) and the effect of its withdrawal after 96 h on hepatocyte insulin receptor status in normal mice

Mice	Plasma metformin (μg/ml)[a]	Total receptor number (per cell × 10^5)	High affinity receptor number (per cell × 10^5)	Low affinity receptor number (per cell × 10^5)	Apparent affinity receptor constant (nmol/l)
Control	ND	3.58 ± 0.23	1.07 ± 0.10	2.50 ± 0.05	2.06 ± 0.10
Metformin 24 h	0.41	4.40 ± 0.38	1.15 ± 0.10	3.24 ± 0.32[b]	1.95 ± 0.20
Metformin 48 h	0.22	4.68 ± 0.19[b]	1.54 ± 0.21	3.14 ± 0.09[b]	1.94 ± 0.13
Metformin 72 h	0.23	4.56 ± 0.18[b]	1.29 ± 0.17	3.26 ± 0.22[b]	1.89 ± 0.06
Metformin 96 h	0.34	5.97 ± 0.76[b]	1.39 ± 0.13	4.58 ± 0.73[b]	1.83 ± 0.07
Metformin withdrawn 24 h	ND	6.00 ± 0.88[b]	1.51 ± 0.20	4.33 ± 0.95	2.04 ± 0.11
Metformin withdrawn 48 h	ND	4.42 ± 0.42	1.30 ± 0.13	3.11 ± 0.38	1.84 ± 0.08

Results are expressed as mean ± SEM of four mice
[a] Analysis performed on pooled 50 μl samples from four mice
[b] $p < 0.05$ compared with control group
ND = not detectable

Table 3. Chronic effect of metformin (120 and 240 mg·kg^{-1}·day^{-1} for 4 weeks) on hepatocyte insulin receptor status in obese diabetic (ob/ob) mice

Mice	Body weight (g)	Plasma glucose (mmol/l)	Plasma insulin (ng/ml)	Total receptor number (per cell × 10^5)	Total receptor concentration (per μm^2 cell surface area)	High affinity receptor concentration (per μm^2 cell surface area)	Low affinity receptor concentration (per μm^2 cell surface area)	Apparent receptor affinity constant (nmol/l)
Lean (+/+) control	40.5 ± 1.1[b]	7.3 ± 0.4[a]	2.1 ± 0.3[b]	3.58 ± 0.23	168 ± 9[b]	48 ± 5[b]	120 ± 2[b]	2.06 ± 0.10
Obese (ob/ob) control	102.7 ± 4.5	11.5 ± 0.3	34.4 ± 1.8	3.44 ± 0.30	106 ± 12	19 ± 3	87 ± 6	1.79 ± 0.10
Obese (ob/ob) metformin (120 mg·kg^{-1}·day^{-1})	96.0 ± 5.8	12.5 ± 0.8	36.6 ± 2.5	4.42 ± 0.26	137 ± 6	25 ± 3	103 ± 12	1.85 ± 0.15
Obese (ob/ob) metformin (240 mg·kg^{-1}·day^{-1})	100.8 ± 4.9	10.1 ± 0.6	21.1 ± 1.6[b]	6.31 ± 0.47[b]	196 ± 12[b]	31 ± 4[a]	162 ± 9[b]	1.83 ± 0.08

Results are expressed as mean ± SEM of four mice
[a] $p < 0.05$
[b] $p < 0.01$ compared with obese control group

teration in the number of high affinity receptors or the apparent affinity constant. When metformin was withdrawn after treatment at a dose of 60 mg·kg^{-1}·day^{-1} for 96 h, the total number of hepatocyte insulin receptors was still raised after 24 h, but values were reduced, approaching control values after 48 h.

Studies in ob/ob Mice

As described elsewhere [7], the ob/ob mice showed gross obesity, moderate hyperglycaemia and severe hyperinsulinaemia (Table 3). The total number of insulin receptors per cell was similar in control lean and ob/ob

Table 4. Chronic effect of metformin (120 and 240 mg·kg^{-1}·day^{-1} for 3 weeks) on the hypoglycaemic response to insulin (100 U/kg, IP) in obese diabetic (ob/ob) mice

Mice	Plasma glucose (percentage decrease)		
	%Δ 15 min	%Δ 30 min	%Δ 60 min
Obese (ob/ob) control	−21.7±0.4	−29.5±0.7	−33.9±2.2
Obese (ob/ob) metformin (120 mg·kg^{-1}·day^{-1})	−18.1±1.6	−27.8±1.5	−27.2±2.6
Obese (ob/ob) metformin (240 mg·kg^{-1}·day^{-1})	−19.3±2.4	−23.8±4.0	−35.9±4.0

Results are expressed as mean±SEM of four mice
%Δ is the percentage change in the plasma glucose concentration compared with time zero

mice. However, as noted previously [12] the size of hepatocytes was greater in ob/ob mice. The diameters were 26±1 and 32±2 μm (mean±SEM, $p<0.05$) in lean and ob/ob mice, respectively. Thus the concentration of receptors in ob/ob mice was substantially reduced when expressed per unit surface area of plasma membrane. This was attributable to a decrease in the concentration of both high (60% decrease) and low (27% decrease) affinity receptors. There was no change in the apparent affinity constant.

Treatment with metformin (120 and 240 mg·kg^{-1}·day^{-1} for 4 weeks) did not alter body weight or plasma glucose concentrations significantly. However, the larger dose of metformin reduced plasma insulin concentrations. This reduction was accompanied by an increase in the total concentration of insulin receptors, due largely to an increase in the low affinity receptors. Treatment with metformin did not alter the apparent affinity constant.

Insulin hypoglycaemia tests in ob/ob mice revealed no significant changes in the hypoglycaemic response to exogenous insulin (100 U/kg) after metformin treatment (Table 4). However, it must be noted that these in vivo tests may not discriminate small changes in insulin action in mice with such pronounced insulin resistance [20].

Chronic Studies in Streptozotocin-Diabetic Mice

Streptozotocin-diabetic mice displayed hyperglycaemia without significant weight loss (Table 5). The total number of hepatocyte insulin receptors was increased compared with age-matched normal mice and was accounted for mainly by an increase in the number of low affinity receptors. Similar observations have been made using several cell types in streptozotocin-diabetic rats [21–26]. In the present study, there was a small decrease in the apparent affinity constant of insulin binding to hepatocytes of streptozotocin-diabetic mice. Treatment with metformin (60 mg·kg^{-1}·day^{-1}) for 10 weeks did not significantly alter body weight, plasma glucose concentrations or hepatocyte insulin receptor status. However, insulin hypoglycaemia tests showed a greater hypoglycaemic response to exogenous insulin (0.15 U/kg) in metformin-treated-streptozotocin-diabetic mice (Table 6).

Table 5. Chronic effect of metformin (60 mg·kg^{-1}·day^{-1} for 10 weeks) on hepatocyte insulin receptor status in streptozotocin-diabetic mice

Mice	Body weight	Plasma glucose	Total receptor number	High affinity receptor number	Low affinity receptor number	Apparent receptor affinity constant
	(g)	(mmol/l)	(per cell × 10^5)	(per cell × 10^5)	(per cell × 10^5)	(nmol/l)
Normal age-matched	42.3±1.9	7.0±0.3	3.5±0.2	1.07±0.18	2.50±0.05	2.06±0.10
Streptozotocin (control)	39.9±2.1	20.2±3.5[a]	6.0±0.3[a]	1.77±0.62	4.21±0.38[a]	1.51±0.14[a]
Streptozotocin+metformin	38.0±1.7	17.3±3.5[a]	6.3±1.1[a]	1.42±0.17	4.95±1.20[a]	1.82±0.31

Results are expressed as mean±SEM of five mice
[a] $p<0.05$ compared with normal age-matched mice

Table 6. Chronic effect of metformin (60 mg·kg^{-1}·day^{-1} for 9 weeks) on the hypoglycaemic response to insulin (0.15 U/kg, IP) in streptozotocin-diabetic mice

Mice	Plasma glucose (percentage decrease)			
	%Δ 15 min	%Δ 30 min	%Δ 60 min	%Δ 90 min
Streptozotocin (control)	−13.1±3.9	−20.3±3.7	−26.1± 4.4	−9.2± 3.9
Streptozotocin+metformin	−36.0±4.5[a]	−45.3±6.2[a]	−32.3±10.3	−19.3±11.4

Results are expressed as mean±SEM of five mice
%Δ is the percentage change in the plasma glucose concentration compared with time zero
[a] $p<0.01$ compared with control group

Discussion

The results demonstrate a rapid (within 24 h) and sustained (up to 50 weeks) increase in the number of low affinity hepatocyte insulin receptors during treatment with metformin ($60\,mg \cdot kg^{-1} \cdot day^{-1}$) in normal mice. This increase corresponds with the effect of metformin on insulin receptors in erythrocytes of non-diabetic and non-insulin dependent diabetic patients [4, 27]. Metformin did not produce a hypoglycaemic effect in normal mice, confirming previous observations in non-diabetic animals and man [1, 8, 28].

In ob/ob mice, a very high dose of metformin ($240\,mg \cdot kg^{-1} \cdot day^{-1}$ for 4 weeks) increased the concentrations of high and low affinity receptors. This effect was accompanied by a small decrease in plasma insulin concentrations, but no significant change in glycaemia. Although this suggests a slight improvement of insulin resistance, insulin hypoglycaemia tests were not altered significantly. A post-receptor defect of insulin action has been noted in ob/ob mice [29, 30]. The present study suggests that this is not corrected by metformin treatment.

Streptozotocin diabetes is associated with hypoinsulinaemia [26], and the increased number of insulin receptors in streptozotocin-diabetic mice may reflect the lifting of a down regulation effect exerted by normal insulin concentrations [17]. At the dose used ($60\,mg \cdot kg^{-1} \cdot day^{-1}$), metformin did not increase the number of hepatocyte insulin receptors significantly in streptozotocin-diabetic mice. However, the number of receptors in these mice was similar to that observed after chronic metformin treatment in normal mice. It is tempting to speculate that this may represent an upper limit for the number of hepatocyte insulin receptors.

Despite no significant effect of metformin on hepatocyte insulin receptor status in streptozotocin-diabetic mice, metformin improved the hypoglycaemic response to exogenous insulin in these mice. Since there is evidence for a post-receptor defect of insulin action in streptozotocin diabetes [26], the present study suggests that this defect is different from the post-receptor defect in ob/ob mice. Metformin improved the hypoglycaemic action of insulin without alteration of hepatocyte receptor status in streptozotocin-diabetic mice. In ob/ob mice metformin produced a substantial increase in hepatocyte receptor number but an effect on insulin action was equivocal. However, the receptor data refer only to hepatocytes, and it is not established whether all insulin target tissues manifest the same defects of receptor and post-receptor status, or respond to insulin in the same manner [31].

The mechanisms responsible for the metformin-induced increase in the number of low affinity hepatocyte insulin receptors in normal and ob/ob mice are not established. However, studies in vitro indicate that metformin can act directly on the target cells without a requirement for de novo protein synthesis [4, 5]. The poor correlation between changes in hepatocyte insulin receptor status and the hypoglycaemic action of insulin during metformin treatment, suggests that metformin might influence post-receptor sites of insulin action independently of effects on insulin receptor binding.

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