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Malarial toxic antigens synergistically enhance insulin signalling

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Hypoglycaemia is a major complication of severe malaria [(1990) Trans. Roy. Soc. Trop. Med. 84 (suppl. 2) 1–65], especially cerebral malaria, in which it is associated with increased mortality [(1990) Lancet 336, 1039–1043; (1989) Quart. J. Med. (New series) 71, 441–459]; however, the mechanisms responsible have not been fully explained. Preparations containing toxic malaria antigens (TMA) released by blood stage *Plasmodium yoelii* malaria parasites have been shown to induce hypoglycaemia in mice lasting at least 8 h [(1992) Clin. Exp. Immunol. (in press)]. Here we report that TMAs can act synergistically with insulin in both stimulating lipogenesis and inhibiting lipolysis in rat adipocytes in vitro, and, furthermore, that they act synergistically with insulin in the induction of hypoglycaemia in vivo.

Adipocyte; Blood glucose; Insulin; Lipogenesis; Lipolysis; Malaria

1. INTRODUCTION

Hypoglycaemia is a major complication of falciparum malaria, particularly in children and pregnant women [1], and is associated with increased mortality in patients with cerebral malaria [2,3], it is also a risk factor for residual neurological sequelae in treated patients [5]. Several suggestions have been put forward as to the possible cause of hypoglycaemia in malaria, including increased glucose consumption by both host and parasite, reduced levels of liver glycogen, impaired hepatic gluconeogenesis, and the production of cytokines, such as tumor necrosis factor (TNF), interleukin-1, lymphotoxin, and interleukin-6 [1,6]. We have recently shown that toxic malaria antigens (TMA) released by blood stage *Plasmodium yoelii* induce hypoglycaemia in mice [4]. Similar, if not identical, antigens have previously been extensively characterized by means of their TNF-inducing capabilities both in vivo in mice and in vitro on macrophages [7,8], however, a monoclonal antibody which neutralizes the cytotoxicity of TNF did not prevent the induction of hypoglycaemia [4]. These results suggested to us that the hypoglycaemia might be due to the antigens acting other than through the induction of TNF. We therefore looked for a direct effect of the antigens on metabolism by measuring their ability to induce lipogenesis or inhibit lipolysis in rat adipocytes in vitro. In this paper we show that TMAs can synergistically enhance insulin effects both on lipogenesis and lipolysis. In addition, a synergistic effect between insulin and TMAs in vivo has been demonstrated.

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2. MATERIALS AND METHODS

2.1. *Toxic malaria antigens*

TMAs were prepared as described previously [4] using the YM lethal variant of *Plasmodium yoelii*. Washed, parasitised erythrocytes with more than 50% parasitaemia were incubated in RPMI culture medium (Gibco, pH 7.3) at 10^6 parasitised cells per ml overnight on a roller at 37°C. Next day, supernatants were collected after centrifugation at 500 × *g* for 10 min, boiled for 5 min and centrifuged at 1,300 × *g*. A *Plasmodium falciparum* supernatant was kindly provided by Dr. H.G. Heidrich (Max-Plank Institute, Germany). Spent culture medium from serum-free merozite harvest was lyophilized and reconstituted in sterile phosphate-buffered saline (pH 7.3).

2.2. *Pronase digestion*

In addition 3 of the 7 *Plasmodium yoelii* supernatants were incubated for 24 h at 37°C with 10 mg/ml of pronase E (Sigma), boiled for 5 min, dialysed against phosphate-buffered saline (pH 7.3) and mixed with polymyxin B-agarose (5 mg/ml) and then centrifuged to remove any bacterial endotoxin.

2.3. *Lipogenesis*

Adipocytes were obtained from epididymal adipose tissues of 6-week-old male Sprague–Dawley rats by digestion with collagenase [9]. The cells were then incubated for 1 h at 37°C (gas phase = O_2/CO_2, 95:5) in 4 ml vols. of Krebs–Henseleit bicarbonate medium containing bovine albumin (10 mg/ml), 5 mM [U-^{14}C]glucose (approx. 100,000 dpm/mmol) and the indicated concentrations of bovine insulin with or without 100 μl aliquots of TMA preparations. Incubations were terminated by the addition of propan-2-ol/hexane/0.5 m H_2SO_4 (40:10:1), followed by extraction of lipids into hexane [10], and quantitation by scintillation counting. All values are expressed relative to the basal condition without insulin or TMAs.

2.4. *Lipolysis*

Adipocytes were incubated for 1 h in 4 ml vols. of Krebs–Henseleit bicarbonate medium containing 5 mM glucose, 1 μM noradrenaline,

bovine albumin (40 mg/ml) and purified calf intestine adenosine deaminase (ADase) as indicated. Incubations were terminated by addition of perchloric acid followed by neutralization of acid-soluble extracts [11], which were then assayed for glycerol by an enzymic method [12].

2.5. *Induction of hypoglycaemia in vivo*

Female outbred Tuck mice (7 weeks of age) were injected intraperitoneally with either 0.025 U of insulin, 0.5 ml of TMA or a combination of insulin + TMA. Two separate preparations of antigen were used. Blood glucose was determined from a drop of tail blood using Glucostix and Ames Glucometer (Miles Ltd.).

3. RESULTS

3.1. *Lipogenesis*

The ability of TMAs to mimic some of the actions of insulin in vitro was investigated using adipocytes, since glucose metabolism and lipolysis are highly sensitive to insulin in these cells. A dose-dependent stimulation of lipogenesis was seen in only three of seven antigen preparations alone, but remarkably, all seven of them acted synergistically with insulin. At maximally effective concentrations, insulin alone increased lipogenesis by 1.5–2-fold, yet, when combined with the antigens, the response to higher concentrations of insulin was doubled (Fig. 1). The antigens did not appreciably alter the EC_{50} for insulin, however, which was approximately 10^{-10} M. In three other experiments (results not shown) the antigens actually decreased lipogenesis by 16% yet still acted in synergy with insulin to increase the response to 3×10^{-9} M insulin by 40%. A preparation from the human malaria parasite, *Plasmodium falciparum*, also acted synergistically with insulin to increase lipogenesis by 33% (mean of 2 experiments). Pronase treatment of the *Plasmodium yoelii* antigens did not affect their ability to act in synergy with insulin in vitro or induce hypoglycaemia in vivo [4]. Neither control preparations from uninfected erythrocytes (n = 5) nor bacterial lipopolysaccharide (even at 100 μg/ml) (n = 3), a well-known inducer of TNF and hypoglycaemia, showed any synergy with insulin (data not shown).

3.2. *Lipolysis*

Insulin also inhibits lipolysis in adipocytes when this process has been stimulated by various receptor agonists, e.g. noradrenaline (see Fig. 2 where EC_{50} for insulin = approx. 10^{-10} M). Without modification of the experimental design, no synergy between the TMAs and high concentrations of insulin was detected in this system; this is because lipolysis is almost completely suppressed by the concentrations of insulin at which synergy was seen in the lipogenesis experiments. Suppression can be overcome, however, by the addition of low concentrations of adenosine deaminase (ADase). This enzyme removes adenosine [13,14] (which is endogenous to the adipocyte incubation system [15]), increases responsiveness to insulin [14,16], and causes tonic inhibition of lipolysis through G_i [17,18]. TMA

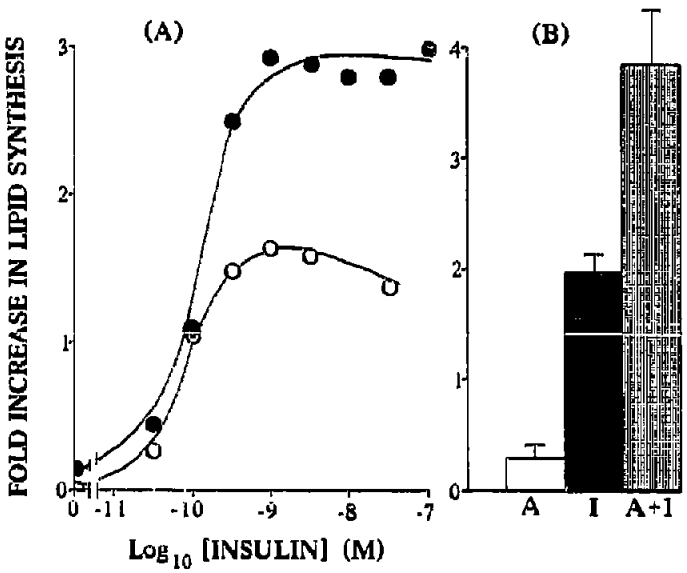

Fig. 1. Toxic malaria antigens (TMA) act synergistically with insulin to increase [^{14}C]glucose incorporation into adipocyte lipids. (A) ○, without TMA; ●, with TMA. The values are means of 2 experiments using 2 separate cell preparations and 2 separate antigen preparations. (B) A, with TMA alone; I, with insulin (3×10^{-9} M); A + I, with TMA and insulin, n = 7 using 7 separate cell preparations and 7 different antigen preparations. Student's t-test indicated $P < 0.05$ for A vs. basal; $P < 0.0005$ for I vs. basal; $P < 0.005$ for A + I vs. I. The bars indicate S.E.M.

preparations alone did not affect noradrenaline-stimulated lipolysis (results not shown), but when the maximal inhibition of lipolysis by insulin was attenuated by ADase at 1 mU/ml, they induced a small, but significant, enhancement of insulin action (Fig. 2). When ADase was present at 3 mU/ml, the effect of insulin alone was even less (−7%), but it was significantly amplified to −25% by the antigens. At 1,000 mU/ml of ADase insulin did not inhibit noradrenaline-stimulated lipolysis (Fig. 2) and no effect of the antigens was observed (results not shown).

3.3. *Effects on blood glucose in vivo*

TMA preparations and insulin also acted synergistically to lower the blood glucose concentration of mice, as is most clearly demonstrated by the experiment summarized in Fig. 3. As previously shown [4] TMAs alone induced hypoglycaemia more slowly than insulin. Whereas injection of insulin of 0.025 U/mouse caused little change in blood glucose, the combination of TMA + insulin acted synergistically to produce a significantly lower blood glucose concentration at 0.5, 1 and 4 h, compared to either TMAs or insulin alone (Student's t-test, $P < 0.01$ in all cases).

4. DISCUSSION

The novel finding from this study is that insulin and toxic malaria antigen (TMA) preparations can act syn-

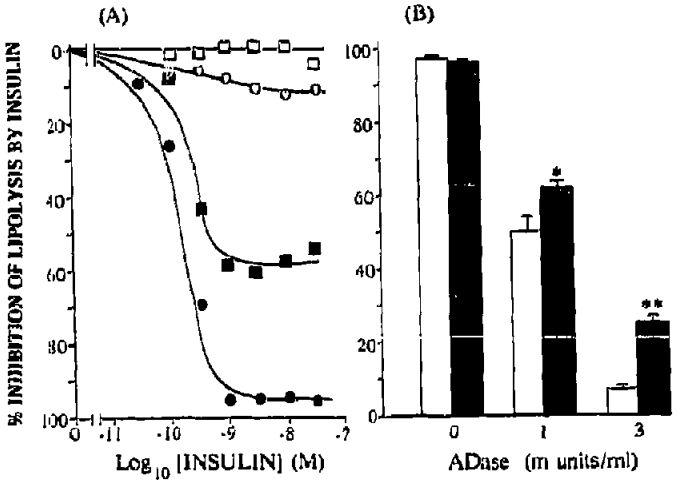

Fig. 2. Toxic malaria antigens (TMA) increase the antilipolytic effect of insulin when low concentrations of adenosine deaminase are present. (A) Insulin-dose–response curves were obtained in the absence of ADase (●); with 1 mU/ml ADase (■); with 3 mU/ml ADase (○), and with 1,000 mU/ml ADase (□), $n = 2$ using 2 separate adipocyte preparations. (B) This demonstrates the percentage inhibition of noradrenaline-stimulated lipolysis achieved by 10^{-8} M insulin at different concentrations of ADase in the absence (open bars) or the presence (filled bars) of 100 ml aliquots of TMA. $n = 4$ using 2 separate cell preparations and 4 separate TMA preparations. $^{*}P < 0.05$ and $^{**}P < 0.001$ for the enhancement of insulin action by the antigen preparations (Student's t-test). The bars indicate S.E.M.

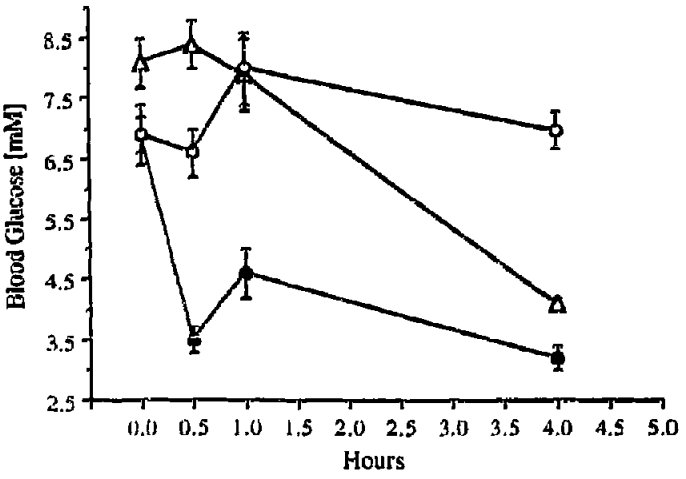

Fig. 3. Synergistic effect of toxic malaria antigens (TMA) and insulin on blood glucose concentrations in mice. The values are means ± S.E.M. of mice injected with 0.025 U insulin (○, $n = 12$); 0.5 ml TMA (△, $n = 8$) or a combination of both insulin and TMA (●, $n = 8$).

ergistically in vivo and in vitro. Although adipocytes were chosen here as a convenient test system, other tissues must be involved in this synergistic interplay between insulin and the antigens in vivo. The amplitude and rapidity of the synergistic effects on blood glucose seen in Fig. 3 are unlikely to be due solely to effects on adipose tissue, and it seems probable that similar synergy occurs in skeletal muscle and/or liver.

The TMAs appear to be phospholipids [4], and the induction of hypoglycaemia in vivo can be competitively inhibited by inositol monophosphate or blocked by antiserum against inositol monophosphate, suggesting that the active component contains an inositol phosphate motif [4]. This structure is also present in inositol phosphate oligosaccharide(s) from mammalian plasma membranes, which mimic insulin action without being synergistic with the hormone [19]. Insulin-like stimulation of glucose metabolism and inhibition of lipolysis have also been reported for a haemodialysate [20] and for a fragment from a *Trypanosoma brucei* glycosylphosphatidylinositol (GPI)-anchored protein [21]. Whilst various other preparations containing an inositol phosphate motif have been shown to have substantial insulin-like effects [22–25], including inhibition of lipolysis [21,26,27]. TMAs appear to be novel in their ability to act in synergy with insulin. In contrast to GPI anchors of parasite antigens which are disrupted by treatment with nitrous acid [28], the ability of TMAs to cause hypoglycaemia is not affected by nitrous acid [4], suggesting differences in structure. As far as we are aware, the only parallel to our findings is the effect of a MHC Class I antigen-derived peptide, which enhances insulin-stimulated glucose uptake by adipocytes, possibly by inhibiting insulin receptor internalization [29].

The clinical picture of hypoglycaemia is often complicated by the hyper-insulinaemic effect of quinine chemotherapy [1,30], or by the recurrence of hypoglycaemia in the absence of hyper-insulinaemia, or by unresponsiveness following dextrose infusion [1,31]. We propose that hypoglycaemia during malaria infection may result from a direct effect of parasite antigens on host tissues, and that the toxic antigens may also exacerbate the hypoglycaemia of patients who have high insulin levels as a result of quinine treatment.

Further characterization of the molecule(s) involved and elucidation of their mechanism of action are required to increase our understanding of the complications of malaria, and for the development of anti-disease vaccines and therapy [32], and possibly also for defining novel substances to decrease insulin resistance in Type II diabetes and other clinical situations.

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