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Lipopolysaccharide-induced insulin resistance in monolayers of cultured hepatocytes

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Summary. In order to clarify the endotoxin effect on the hepatic removal of insulin, the influence of lipopolysaccharide (LPS) from *E. coli* O111:B4 on the insulin binding and endocytosis in cultured hepatocytes from adult male rats has been investigated. LPS decreases both processes in a time and temperature-dependent manner, showing a major effect at short time and low temperature, according to the characteristics of LPS binding and uptake.

Keywords: endotoxic shock, lipopolysaccharide (*E. coli*), hepatocyte cultures, insulin binding, insulin resistance, insulin internalization

It is well known that endotoxaemia exerts a major impact on metabolic requirements, and that some metabolic–endocrine derangements may represent the primary defects in this type of shock.

During severe endotoxic shock, liver function is impaired with a subsequent depression of hepatic gluconeogenesis (Filkins & Cornell 1974; Groves *et al.* 1974; Cerra *et al.* 1979). Thus, the supply of available glucose is often less than that required for normal metabolism. On the other hand, abnormal insulin levels have been reported (Clowes *et al.* 1974; Hinshaw *et al.* 1975) as well as ineffectiveness of insulin for the uptake of glucose. Increased insulin resistance in some tissue has also been described (Clowes *et al.* 1978).

Primary steps of endotoxicosis are somehow mediated by the direct interaction of LPS with cell membranes. As we have previously shown, LPS from *Escherichia coli* O111:B4 binds to the hepatocyte membrane by means of hydrophobic and electrostatic non-specific interactions (Pagani *et al.* 1981). This fact has been related to an increase of membrane microviscosity (Portolés *et al.* 1987).

Following the initial binding of [^{125}I]insulin to the surface of isolated hepatocytes, the hormone is progressively internalized by the cell in a time and temperature-dependent manner (Olefsky & Kao 1982; Poole *et al.* 1982). The uptake may serve as a means of down-regulating the insulin-receptor concentration (Krupp & Lane 1981; Draznin *et al.* 1984).

In an attempt to clarify the molecular mechanisms involved in the hormonal alterations induced by the endotoxin at cellular

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level, the influence of LPS on molecular bases of insulin action has been studied using hepatocytes in monolayer cultures.

Materials and methods

Materials

Lipopolysaccharide from *E. coli* O111:B4, obtained according to Westphal's method (Westphal *et al.* 1952), was supplied by Difco (Detroit, MI, USA). Unlabelled insulin was obtained from Novo España SA (Spain). Monoiodinated [^{125}I]insulin (30 μCi/μg) was purchased from Amersham International (UK). William's E medium was obtained from Flow Laboratories (Irvine, Ayrshire, UK), penicillin and streptomycin from Antibioticos SA (Spain) and foetal calf serum from Gibco Europe (Paisley, Renfrewshire, UK). Bovine serum albumin and collagenase (type I) were from Sigma Chemical Co (St Louis, MI, USA). Other chemical reagents were purchased from Merck (Darmstadt, FRG).

Animals

Male Wistar rats (150-200 g bodyweight), fasted over 20 h with water *ad libitum*, were used in all the experiments.

Isolation and culture of hepatocytes

Hepatocytes were isolated by the perfusion technique, using collagenase in Krebs–Ringer bicarbonate solution (KRB-medium), according to the method of Berry and Friend (1969) as described previously (Pagani *et al.* 1981; Portolés *et al.* 1987).

Yield of viable hepatocytes was $(100 \pm 20) \times 10^6$ per rat liver. Cell viability, determined by the trypan blue exclusion test, was 85 to 95%.

The isolated hepatocytes, cultured in William's E medium and in the presence of 10% foetal calf serum, were grown at 37°C in tissue culture plates (24 flat-bottomed wells) (3×10^5 cells/well) with circular glass microcoverslips, under CO_2/O_2 (5%:95%) atmosphere for 24 h until the formation of a continuous monolayer. Cell culture was performed under sterile conditions in a laminar flow chamber, Telstar CAM 7001.

Insulin-binding and internalization assays

Hepatocyte monolayers, on glass circular micro-coverslips, were washed with William's E medium (500 μl) and preincubated in either the presence or the absence of LPS (100 μg/3×10^5 cells/500 μl William's E medium) during 30 min at 25°C, under CO_2/O_2 (5%:95%) atmosphere. The medium was then aspirated and the cells were washed with William's E medium (500 μl) and incubated with a constant concentration of [^{125}I]insulin (400 pg/3×10^5 cells) in either the presence or the absence of increasing concentrations of unlabelled hormone according to the experiment. Incubations were carried out in William's E medium with albumin (1%) for varying times and at different temperatures (4, 25 and 37°C) under CO_2/O_2 (5%:95%) atmosphere. The assay was stopped by aspirating incubation medium and washing monolayers with William's E medium (500 μl) containing albumin (1%).

The internalization of [^{125}I]labelled insulin was determined by the acidification technique (Olefsky & Kao 1982). Cell-associated radioactivity was extracted with acid by a modification of the method of Haigler *et al.* (1980) to remove material bound to the cell surface. After stopping incubation and adding William's E medium (500 μl) with albumin (1%), monolayers were incubated with 2 ml of barbital sodium acetate buffer (pH 3·0), containing 28 mM Na acetate, 20 mM Na barbital and 117 mM NaCl, at 4°C for 6 min.

The acid-extractable radioactivity measures the surface-bound insulin, and the non-acid-extractable radioactivity corresponds to the internalized hormone. Internalized plus surface-bound insulin gives the total specific associated hormone.

In these experiments, non-specific binding

is defined as the amount of [^{125}I]insulin remaining in the indicated fractions (total cell-associated radioactivity, non-acid-extractable radioactivity and acid-extractable radioactivity), in the presence of a great excess (50 μg/3 × 10^5 cells/500 μl medium) of unlabelled insulin.

Microcoverslips were transferred to plastic tubes for counting in a Beckman counter (Gamma 5500) to estimate the cell-associated radioactivity.

Statistics

All assay points were performed in triplicate. Data given in figures and tables represent the mean values of three determinations ± standard deviations (s.d.).

Results

In order to study the influence of lipopolysaccharide from *E. coli* O111:B4 on the binding of insulin to cultured hepatocytes, monolayers were preincubated with endotoxin previously to the addition of the hormone. The non-specific binding was estimated as the amount of [^{125}I]insulin bound to cells in the presence of a great excess of unlabelled insulin. Subtraction of non-specifically bound hormone from the total radioactive uptake generated specific binding curves. As shown in Fig. 1, preincubation with lipopolysaccharide decreases the insulin specific binding at every hormone dose assayed.

This decrease was dependent on the endotoxin dose (unpublished results), and the relatively high amount of LPS (pharmacological dose) used in the assays is justified to obtain clear results within the time scale of the experimental models.

Table 1 shows the effect of preincubation of hepatocyte cultures with LPS on the [^{125}I]insulin specific binding at different temperatures and after 90 min incubation with the hormone. Values are given as percentages of the insulin binding to the control cells at every temperature assayed. The LPS-induced decrease on the insulin binding is

Table 1. Effect of LPS on the insulin specific binding at different temperatures. Cultured hepatocytes (3 × 10^5 cells/500 μl William's E medium) were preincubated with or without LPS (100 μg/3 × 10^5 cells) during 30 min at 25°C; medium was aspirated and cells were then incubated with the labelled hormone (400 pg/3 × 10^5 cells) at different temperatures. Non-specific binding was estimated as indicated under Materials and methods. Values are given as percentages of the control cells at every temperature assayed

t(°C)	4	25	37
B(%)	41 ± 7	70 ± 14	100 ± 9

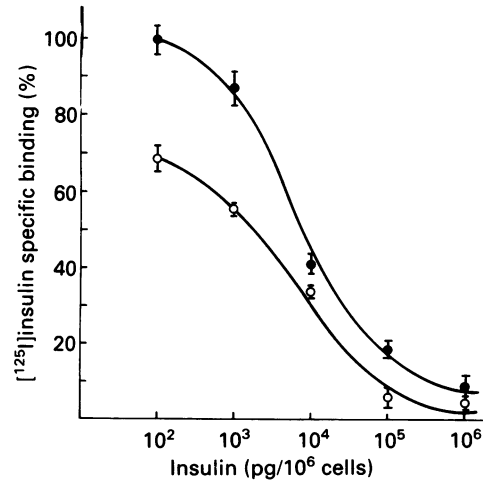

Fig. 1. Effect of LPS on insulin uptake by cultured hepatocytes. Cultured hepatocytes (3 × 10^5 cells/500 μl William's E medium) were preincubated with (○) or without (●) LPS (100 μg/3 × 10^5 cells) during 30 min at 25°C; medium was aspirated and cells were then incubated with a constant concentration of [^{125}I]insulin (400 pg/3 × 10^5 cells) and increasing concentrations of unlabelled hormone (0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 pg/10^6 cells) for 90 min at 25°C. Values are percentages of specific binding of [^{125}I]insulin referred to the binding control of the labelled hormone to the cells in the absence of unlabelled native insulin.

more evident at 4, lower at 25, and disappears at 37°C.

In order to know the LPS effect on the insulin internalization and its time and temperature dependence, various studies were carried out using the acid extraction technique.

Figure 2 shows the total specific insulin association, the surface-bound insulin, and its internalized state in cultured hepatocytes preincubated with LPS and incubated with [^{125}I]insulin during different times at 37°C.

At short incubation times (30 min) LPS clearly decreases the contribution of both surface-bound insulin (acid extractable bound hormone) and internalized insulin to the total insulin association. As incubation proceeds, total insulin association is less affected by endotoxin, whereas the LPS effect on the surface-bound insulin disappears and even becomes higher than control values. On the other hand, a significant decrease of insulin internalization produced by LPS is observed at all incubation times.

The temperature dependence of this effect of LPS on the internalization of insulin was investigated with hepatocyte cultures preincubated with endotoxin and incubated with the hormone. Figure 3 shows that the decrease of insulin internalization induced by LPS is more evident at low temperatures (4°C) than at 37°C.

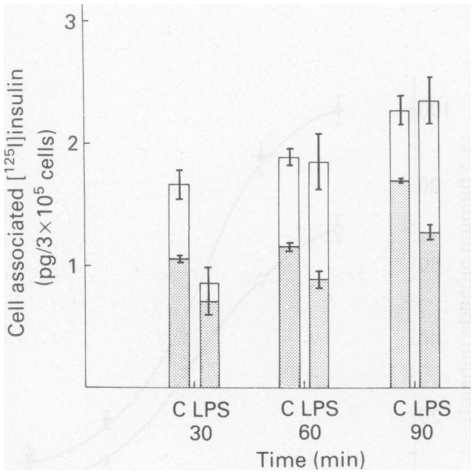

Fig. 2. Effect of LPS on binding and internalization of insulin at different times. Cultured hepatocytes (3×10^5 cells/500 μl William's E medium) were preincubated with or without LPS (100 μg/3×10^5 cells) during 30 min at 25°C; medium was aspirated and cells were then incubated during different times (30, 60 and 90 min) at 37°C with a constant concentration of [^{125}I]insulin (400 pg/3×10^5 cells) either in the presence or the absence of a great excess (50 μg/3×10^5 cells) of unlabelled hormone. Medium was aspirated and internalization of labelled insulin was determined by the acidification technique. C, Control values; LPS, cells treated with LPS; □, surface-bound insulin; ▨, internalized insulin. Internalized plus surface-bound insulin gives the total specific associated hormone.

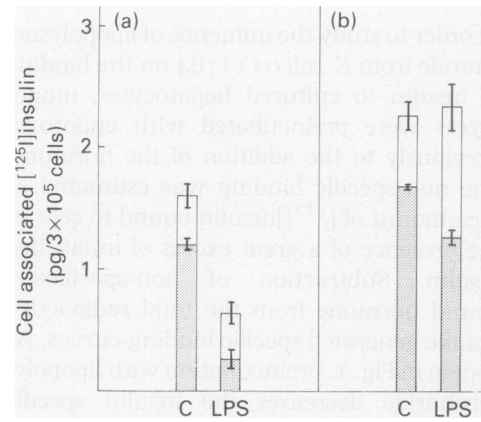

Fig. 3. Effect of LPS on binding and internalization of insulin at different temperatures. Cultured hepatocytes (3×10^5 cells/500 μl William's E medium) were preincubated with or without LPS (100 μg/3×10^5 cells) during 30 min at 25°C; medium was aspirated and cells were then incubated during 90 min at different temperatures (a, 4°C; b, 37°C) in the presence or the absence of a great excess (50 μg/3×10^5 cells) of unlabelled hormone. Medium was aspirated and internalization of labelled insulin was determined by the acidification technique. C, Control values; LPS, cells treated with LPS; □, surface-bound insulin; ▨, internalized insulin. Internalized plus surface-bound insulin gives the total specific associated hormone.

Discussion

Severe derangements in metabolic homeostasis are prominent components in the physiologic response to endotoxicosis. The metabolic responses to endotoxin include a transient, early hyperglycaemia followed by a progressive hypoglycaemia and a depletion of body carbohydrate reserves (Hinshaw 1976). A decrease in hepatic gluconeogenesis and an increased peripheral tissue utilization of glucose underwrite the profound hypoglycaemia, which has been related to the transition from reversible endotoxicosis to lethal endotoxic shock. These metabolic events are characteristic of hyperinsulinaemia and for this reason insulin has been involved as key factor in the glucose dyshomeostasis of endotoxicosis (Yelich & Filkins 1980). Hyperinsulinaemia has been shown during endotoxicosis (Blackard *et al.* 1976; Spitzer *et al.* 1976) although the mechanism responsible for the elevated insulin levels is not completely understood. Evidence exists for a monokine (interleukin I) involvement in post-septic hyperinsulinaemia as related to a hypersecretion state of the pancreas (Yelich & Filkins 1982), supporting the importance of some serum mediators in the development of endotoxic shock. On the other hand, forms of insulin resistance appear to be present during the hyperdynamic phase of sepsis (Stoner *et al.* 1983).

In order to clarify the endotoxin effect on hepatic removal of insulin, the influence of bacterial lipopolysaccharide on various steps of the mechanism of insulin action in cultured hepatocytes, has been investigated.

Studies on the binding of [^{125}I]insulin to cultured hepatocytes showed the typical competition curve (Fig. 1) as reported for insulin in different cell types and plasma membrane preparations (Donner 1982; Olefsky & Kao 1982).

The endotoxin interaction with monolayers decreases the specific insulin binding at all hormone doses assayed (Fig. 1), after 90 min incubation at 25°C. These data are in agreement with those previously obtained using isolated hepatocytes in suspension (Pagani *et al.* 1984). These results demonstrate that the binding of LPS to the hepatocyte membrane does not allow normal membrane interaction with the hormone. This effect has been investigated at different times and temperatures of incubation with insulin. The LPS action on the hormone binding was more evident at 30 min and low temperatures (4°C) (Table 1, Figs 2 and 3). However, the effect of LPS on hormone binding disappears at higher time intervals (90 min) and temperatures (37°C).

We have previously shown that LPS binds to particular ingredients of the membrane bilayer in a non-specific manner (Pagani *et al.* 1981), inducing an increase in microviscosity which has been observed in both hepatocyte suspension and monolayers (Portolés *et al.* 1987). The possible influence of these physical changes in the membrane on insulin uptake was estimated.

Insulin internalization was investigated at several temperatures and times showing that endocytosis is a time and temperature-dependent process (Figs 2 and 3), in accord with other results (Ose *et al.* 1980; Olefsky & Kao 1982). Insulin endocytosis and LPS internalization are delayed at low temperatures but are still active at 4°C as is shown by 90 min incubation with insulin (Fig. 3) and Au-LPS immunocytochemical studies (Municio *et al.* 1988).

On the other hand, our results showed that LPS exerts a significant decrease in insulin internalization in all conditions included in our experimental design (Figs 2 and 3). Thus, when insulin binding is less affected, at 37°C and 90 min, the LPS effect on insulin internalization was observed to be permanent.

These results are in agreement with the properties of the LPS binding and uptake. These processes are time and temperature-dependent so that endotoxin remains on the cell surface at low temperatures and short times as has been observed using immunocolloidal gold technique (Municio *et al.* 1988). The LPS binding increases at longer

times and higher temperatures, and a progressive internalization of the endotoxin is then observed. So, when the incubation time is short or the incubation temperature is low (conditions which delay LPS uptake), the endotoxin affects insulin binding and internalization. When the endotoxin uptake by the cells proceeds intensively (higher temperatures and longer times), the hormone binding was less affected; however, insulin endocytosis remains altered by LPS under these conditions. The increase of microviscosity in the parenchymal cell membrane produced by the direct action of LPS (Portolés *et al.* 1987), could alter both the endocytic process and the mechanism(s) whereby insulin triggers the intracellular response. Using different techniques, it has been demonstrated that hormone receptors were able to migrate independently in the membrane lipid bilayer and regulate membrane enzymes through collisions between these components and guanine nucleotide regulatory subunits. These interactions can play a key role in the modulation of some enzymatic activity, e.g. the adenylate cyclase system (Houslay 1986). We have observed that LPS treatment alters the glucagon-induced synthesis of cyclic AMP (Pagani *et al.* 1984). Although further studies are needed for understanding the effect of endotoxin on hormone secretion and action mechanisms, the results presented here support the hypothesis that LPS produces the insulin resistance observed in endotoxicosis through a direct action that involves both insulin binding to receptors and hormone-receptor internalization.

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