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EFFECT OF LIPID EXTRACTION, ADRENALECTOMY, AND CORTISOL UPON FREE RADICAL FORMATION BY RAT LUNG MICROSOMES

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SUMMARY

Free radical formation and superoxide anion production by rat lung microsomes have been determined by measurement of conversion of epinephrine to adrenochrome *in vitro*. There was a marked increase in superoxide anion production by the microsomes following extraction with 10% aqueous acetone as has been seen previously following adrenalectomy in animals exposed to 100% oxygen. When the extracted lipid was added back to the extracted microsomes there was a significant reduction in the superoxide anion production similar to the reduction seen following cortisol administration to adrenalectomized animals.

INTRODUCTION

Reports from this laboratory have described an effect of cortisol to decrease free radical production leading to epinephrine to adrenochrome conversion by rat lung microsomes [1–3]. Many studies have shown an influence of corticosteroids upon the phospholipid composition of the lung [4, 5], and also a phospholipid requirement for the NADPH cytochrome *c* reductase of rat liver microsomes and many other lipid activated enzymes [6–8]. An effect of corticosteroids upon the phospholipid composition of fat cell ghosts obtained from rat epididymal fat pads which parallels the inhibitory action of corticosteroids on glucose transport has been demonstrated [9]. Changes in the phospholipid composition of leukocytes obtained after treatment of normal human subjects with cortisol or dexamethasone have also been reported [10]. It has been postulated, based on these and other studies, that changes in the phospholipids of membranes mediate some of the effects of the corticosteroids. If an alteration in protein-lipid relationships is responsible for the effect of adrenalectomy and corticosteroids upon free radical formation by rat lung microsomes, extraction of lipid from the microsomes might produce changes in activity which could be related to the hormonal effects. If corticosteroids increase specific phospholipids of rat lung microsomes as they do those of leukocytes and fat cell ghosts, extraction of the lipid might stimulate free radical production and readdition of the phospholipid might decrease free radical production. With these possibilities in mind free radical production leading to epinephrine to andrenochrome conversion by rat lung microsomes has been studied before and after lipid extraction of the microsomes and following addition of the extracted lipid back to the microsomes.

EXPERIMENTAL

Methods were essentially as previously employed [3]. Female Sprague–Dawley rats weighing 150–200 g were guillotined. Both lungs were immediately removed, placed in ice cold 0.3 M sucrose, cut into small pieces, and homogenized with 20 or more strokes of a Ten-Broeck homogenizer. The homogenate was centrifuged at 2,000 *g* for 10 min and the supernatant at 12,500 *g* for 10 min. The supernatant from the latter spin was centrifuged at 105,000 *g* for 60 min and the microsomal pellet resuspended in 2 ml of 0.3 M sucrose for use in the incubation.

Free radical (superoxide anion) production was determined by measuring epinephrine conversion to adrenochrome as described by Aust *et al.*[11]. Incubations were performed in cuvettes containing 10^{-4} M EDTA, 10^{-4} M NADPH in 0.15 M potassium phosphate buffer, pH 8.5, and microsomes (microsomal protein averaged 0.1 mg/ml) in a total volume of 3.0 ml. The baseline was balanced in an Aminco-Chance spectrophotometer in the split beam mode. The reaction was started by addition of 5×10^{-4} M epinephrine to one of the cuvettes. The absorption change at 480 nm was followed at 5 min intervals until the reaction ceased. Autooxidation, in the absence of NADPH, did not occur under the conditions of the experiment. The formation of adrenochrome was largely blocked by the addition of superoxide dismutase. Nanomoles adrenochrome formed were calculated using a molar extinction coefficient of 4020 [12]. Microsomes were extracted with 10% aqueous acetone for removal of the lipid-phospholipid fraction as described by Lester and Fleischer[13] and employed by Jones and Wakil[6]. Lipid extracted in this manner from similarly prepared microsomes was used in the lipid addition experiments. Incuba-

tions were then performed with whole and lipid extracted microsomes and with lipid extracted microsomes to which were added previously extracted microsomal lipid which was sonicated for 4 min with a 200 Watt sonicator run at 70% power. Cortisol was administered 1–4 days following adrenalectomy as 2.5 mg of Cortef (The Upjohn Co., Kalamazoo, Michigan) subcutaneously at each of two different sites. This was repeated 24 h later and on the third day 1 mg of cortisol sodium succinate (Solu-Cortef, The Upjohn Co., Kalamazoo, Michigan) was administered subcutaneously 1 h prior to sacrifice. Corticosterone was determined by a fluorometric procedure [14]. Protein was determined by the method of Lowry *et al.*[15]. Superoxide dismutase was obtained from Diagnostic Data Inc., Mountain View, California. Statistics were performed using Student's *t*-test [16].

RESULTS

Figure 1 illustrates the effect of aqueous acetone extraction of microsomes obtained from intact animals upon production of adrenochrome from added epinephrine. Extraction of the microsomes with 10% aqueous acetone resulted in a significant increase in the amount of adrenochrome formed (179 ± 42.1 nmol/mg microsomal protein to 341 ± 62.7 SE, $P < 0.025$). Addition of sonicated lipid extract to different aliquots of the same extracted microsomes considerably reduced epinephrine to adrenochrome conversion (341 ± 62.7 nmol to 204 ± 27.5, $P < 0.025$). No adrenochrome formation occurred in the absence of added NADPH thus demonstrating the need for an electron donor in the formation of the adrenochrome. In like manner, addition of less NADPH (10^{-5} or 10^{-6} M) resulted in considerably less adrenochrome formation although higher concentrations of the electron donor did not result in greater epinephrine conversions than those shown.

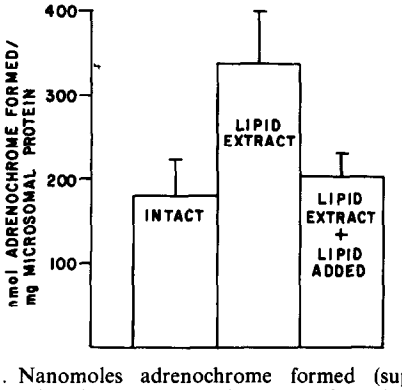

Fig. 1. Nanomoles adrenochrome formed (superoxide anion produced) when lung microsomes from intact rats were incubated with NADPH and epinephrine. Extraction of the microsomes by 10% aqueous acetone (lipid extract) resulted in a significant increase in superoxide anion production. Addition of the extracted lipid back to the microsomes (lipid extract plus lipid added) suppressed superoxide anion production and returned it to essentially the same level as that of the whole microsomes. Mean of eight experiments with standard error of the mean is shown.

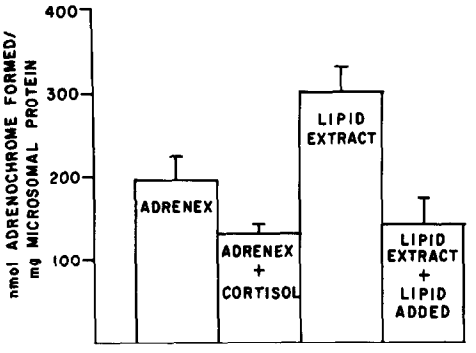

Fig. 2. Nanomoles adrenochrome formed (superoxide anion produced) when lung microsomes from adrenalectomized rats and cortisol treated adrenalectomized rats were incubated with NADPH and epinephrine as described in methods. Mean of 15 experiments with standard error of the mean is shown. There was a significant increase in superoxide anion production by microsomes of adrenalectomized animals when the lipid was extracted with 10% aqueous acetone (lipid extract) and a significant decrease in production of the free radical when the extracted lipid was added back to the microsomes (lipid extracted plus lipid added).

Figure 2 illustrates the effect of a similar experiment using lung microsomes obtained from adrenalectomized rats. As reported previously in a much larger series [3] treatment of the animals with cortisol for 48 h prior to preparation of the microsomes resulted in a significant decrease of free radical formation. Extraction of lipid from microsomes with 10% aqueous acetone produced a significant increase in adrenochrome formation (196 ± 28.0 nmol mg microsomal protein to 302 ± 27.8, $P < 0.025$) and adding the lipid back to the extracted microsomes significantly suppressed the adrenochrome formation to 143 ± 28.8 nmol ($P < 0.001$).

Thus, lung microsomes obtained from both intact and adrenalectomized animals increase their conversion of epinephrine to adrenochrome after extraction with aqueous acetone, but that increase is reduced when the lipid is added back to the microsomes during incubation with NADPH and epinephrine. The increase in free radical production is similar to that previously reported to occur with rat lung microsomes obtained from adrenalectomized rats exposed to 100% oxygen for 24 h as compared to those obtained from similarly treated intact animals. The decreased production seen on readdition of the extracted lipid is similar to the decreased production following administration of cortisol to adrenalectomized animals. The previously reported differences in adrenochrome formation following cortisol administration must be considered pharmacologic in nature as plasma cortisol levels were 38–48 μg/dl at the time

of decapitation as measured by radioimmunoassay. Corticosterone blood levels in the present study were 24 ± 12 μg/dl in normal "unstressed" animals and decreased to <5 μg/dl following adrenalectomy.

DISCUSSION

The much larger difference in adrenochrome formation between intact and cortisol treated animals than between intact and adrenalectomized is probably related to the greater disparity in plasma steroid levels. A somewhat different effect of cortisol as compared to that of the corticosterone normally secreted by the rat can not be ruled out. Accentuation of the differences between microsomes obtained from intact and adrenalectomized animals by exposure to 100% oxygen may have resulted from the peroxidizing effect of the oxygen upon unsaturated fatty acids in the membrane [3, 17, 18].

The formation of adrenochrome from epinephrine in these studies can not be looked upon as a specific enzymatic reaction. This oxidation is known to be a chain reaction, the stoichiometry of which can not be determined. The requirement of the reaction for an electron donor and its limitation by the amount of NADPH added suggests, however, that the initiation is by NADPH requiring enzymatic activity and not by autooxidation. It was also demonstrated that the chain reaction is terminated in the absence of a continuing supply of the initiator which is presumed to be superoxide anion or other free radical. Inhibition of the reaction by superoxide dismutase shows that superoxide anion is involved in the chain reaction. As previously reported acetylated cytochrome *c* is minimally reduced by the preparation indicating that very small amounts of superoxide anion or free radical act as initiator of the epinephrine to adrenochrome conversion [3]. Use of the adrenochrome reaction appears, therefore, to be a useful means of amplifying the effects occurring in the microsomal preparation but does not give specific information concerning the nature of the free radical produced by the microsomes.

The source of the superoxide anion produced by the rat lung microsomal preparation is not fully known, but rat lung microsomes contain a flavin dehydrogenase and P-450 oxygenase which are capable of producing superoxide anion. Aust *et al.*[11] have shown that the epinephrine oxidation activity of rat liver microsomes copurifies with NADPH-cytochrome *c* reductase and that this enzyme is presumably a source of superoxide anion production by these microsomes. A number of studies have demonstrated the presence of a cytochrome P-450 dependent benzo(α)pyrene monooxygenase in rat lung [19]. Superoxide anion also may be produced in the hydroxylation reactions associated with cytochrome P-450 [20]. H_2O_2 production in liver microsomal fractions has been shown to result from both flavoprotein dependent NADPH oxidation and autooxidation of cytochrome P-450 as evidenced by CO inhibition of approx. one third of the H_2O_2 produced [21]. The formation of superoxide anion on reoxidation of reduced flavin has been demonstrated by the reduction of cytochrome *c* [22] and by catalysis of the oxidation of epinephrine [23].

The marked increase in superoxide anion production observed in rat lung microsomes following lipid extraction reported in this study is suggestive of the uncoupling of oxygenase activity discussed by Hayaishi, in which a modified oxygenase-substrate complex may not hydroxylate but still be reactive with molecular oxygen [24]. Addition of the lipid fraction to the extracted microsomes presumably reestablishes a normal complex and reduces the superoxide anion production. Many investigations have established the relative specificity of the phospholipid requirement for lipid activated enzymes [8]. Phospholipid phase transitions have also been reported to affect the electron transfer from the reductase to cytochrome P-450 in rat liver microsomes [25]. It has been suggested that not only the presence of a phospholipid but the saturation of its fatty acid components determines the efficiency of interaction between cytochrome P-450 and NADPH-cytochrome P-450 reductase [7]. An effect of corticosteroid therapy upon phospholipid synthesis by rat lung microsomes is well known. The action is thought chiefly to influence synthesis of dipalmitoyl phosphatidylcholine (surfactant) which plays a primary role in altering alveolar surface tension. Preliminary studies have demonstrated that the microsomes prepared from cortisol treated animals employed in these studies may have an increased concentration of phosphatidylcholine. It will be necessary to isolate specific cell types to establish that this increase is not secondary to the presence of type II cells which are responsible for the increased production of surfactant. Corticosteroid effects on phospholipids of fat cells and leukocytes, which appear to be related to physiologic functions, raise the possibility that increased amounts of phosphatidylcholine or other phospholipid changes in lung microsomes may also influence reactions known to be important in pulmonary function. This would be consistent with our previous demonstration of effects of corticosteroids on aryl hydrocarbon hydroxylase and cytochrome *c* reductase of rat lung microsomes, which are enzymatic reactions known to be influenced by their lipid environment.

Further investigations will have to be undertaken to determine whether changes in superoxide anion production by rat lung microsomes following adrenalectomy and exposure to 100% oxygen, or administration of cortisol are secondary to the alteration of the phospholipid, or due to other factors. The present studies demonstrate, once again, the importance of a lipid factor in the activity of a microsomal preparation. An increase in superoxide anion production following lipid extraction, similar to that observed previously following adrenalectomy, and a decrease

following readdition of the phospholipid, is consistent with the postulate that some of the effects of the corticosteroids are mediated by their actions to influence synthesis and/or distribution of phospholipids in membranes [9, 10, 26].

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