# Effect of Hydrocortisone on Oxygen Free Radical Generation by Mononuclear Cells

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**Corticosteroids are known to exert antiinflammatory and immunosuppressive effects. Since reactive oxygen species (ROS) induce tissue damage and inflammation and since mononuclear cells (MNCs) generate ROS, we investigated whether corticosteroids inhibit ROS generation by MNCs when given systemically. A single dose of either 300 mg (n = 8) or 100 mg (n = 6) of hydrocortisone (HC) was injected intravenously into eight and six subjects, respectively. Blood samples were obtained before and sequentially after the injection. Following 300 mg HC, *N*-formylmethionyl leucyl phenylalanine (fMLP)-induced ROS generation, assayed by measuring chemiluminescence with luminol, decreased significantly at 0.5 hours and reached a nadir at 2 hours (8% of basal, $P < .001$); thereafter, it gradually recovered, but was still below baseline at 24 hours. Following the dose of 100 mg HC, ROS generation decreased significantly at 1 hour (nadir, 30% of basal; $P < .01$) and gradually recovered to near basal level at 8 hours. Serum cortisol concentrations were markedly elevated over basal and remained elevated throughout the first 8 hours of the experiment, returning to baseline at 24 hours. This inhibition of ROS generation by HC (and other glucocorticoids) may have a role to play in mediating the antiinflammatory action of corticosteroids.**



CORTICOSTEROIDS ARE WELL ESTABLISHED in their clinical use as antiinflammatory drugs and are known to suppress both immunological function and acute inflammatory and allergic responses.[1,2] This has led to their successful use in acute allergic conditions and in autoimmune conditions in which the inflammation is mediated by immunological mechanisms.[3-5] Several possible mechanisms have been suggested as having a role in this antiinflammatory effect of corticosteroids. More recently, it is has been shown that hydrocortisone in vitro may have an inhibitory effect on reactive oxygen species (ROS) generation in leukocytes, in particular neutrophils. Umeki and Soejima[6] have demonstrated that corticosteroids have an inhibitory effect on superoxide radical generation by neutrophils exposed to phorbol myristate acetate (PMA) and that in a cell-free system, hydrocortisone (HC) has an inhibitory effect on NADPH oxidase activated by sodium dodecyl sulfate. The $IC_{50}$ for this effect of hydrocortisone was 40 mmol/L. These observations were confirmed by Roilides et al,[7] who demonstrated that HC at a dose of 30 mmol/L or dexamethasone at a dose of 1 mmol/L significantly inhibited the release of superoxide radical by polymorphonuclear leukocytes in response to *N*-formylmethionyl leucyl phenylalanine (fMLP). In another study, Fukushima et al[8] demonstrated that chronic administration of corticosteroids inhibited fMLP-stimulated superoxide production by polymorphonuclear leukocytes ex vivo. While the well-known effects of corticosteroids in inducing eosinopenia may mediate a part of their antiallergic effect, and the lymphopenic effect may help their antiimmunological actions, it is likely that corticosteroids may exert an important effect by suppressing ROS generation at the inflammatory site in vivo. ROS are known not only to mediate bactericidal activity of phagocytes, but they may also result in localized tissue damage, as in the case of ischemia reperfusion injury, and at other inflammatory sites in general.

Corticosteroids are consistently used in acute asthmatic exacerbations, and a dose of 100 to 300 mg given as a single intravenous bolus is commonly used in such clinical settings. Often, the doses used are much greater, as in regimens for organ transplant rejection, when 1,000 mg methylprednisolone may be given in a single intravenous infusion (equivalent to 5,000 mg of HC). In acute asthma, corticosteroids are known to exert their effect through suppression of eosinophilic activation, IgE-allergen interaction, and suppression of IgE biosynthesis.[2,9-11] While these data show that corticosteroids have an inhibitory effect on superoxide radical generation by leukocytes in vitro, no quantitative data are available about their effect in vivo, nor is any information available about the pharmacodynamic aspects of this effect.

We undertook the present study to elucidate the action of HC given at doses of 100 mg and 300 mg intravenously, two commonly used doses, on ROS generation. We believed that such data would be useful in understanding the action of these drugs in clinical settings, where HC is commonly used.

## MATERIALS AND METHODS

Eight normal subjects (age range, 24 to 50 years; mean, 38.54 ± 8.98; weight range, 60 to 100 kg) volunteered for the study. None of the subjects had a previous history of significant chronic illness, including endocrine disease. None of the subjects had previously taken corticosteroids. None had taken aspirin or other nonsteroidal antiinflammatory drugs for 1 week.

On the morning of the experiments (7 to 8 AM), each subject had an intravenous butterfly needle inserted into the cephalic vein in the forearm or at the wrist. The needle was kept open with heparin. Blood samples (10 mL) were obtained with vacutainers after the initial 2 mL was discarded. Blood was collected in EDTA for blood counts, in heparin for monunuclear cell (MNC) preparation, and in plain glass tubes for HC (cortisol) assays. Subjects were injected intravenously with HC at a dose of either 100 mg or 300 mg. Sequential blood samples were obtained before HC injection and sequentially at 30 minutes, and 1, 2, 4, 8, and 24 hours after the injection.

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Blood samples for cortisol were allowed to clot and were centrifuged. Serum was separated and frozen at −20°C until the time of the assay.

### *Preparation of MNC*

MNCs isolated from heparinized blood (14.3 USP/mL) were fractionated by a density gradient centrifugation over Ficoll (Organon Teknika, Durham, NC; density of 1.077 to 1.080 g/mL at 20°C). Initially, the blood was centrifuged at 235 × *g* for 10 minutes to obtain a buffy coat. The buffy coat was diluted to 3 mL in Hank's balanced salt solution (HBSS) and layered on top of 3 mL Ficoll and respun at 235 × *g* for 30 minutes at 22°C. The mixed MNC band at the interface was removed with a Pasteur pipette, and the cells were washed twice in HBSS. The remainder of the blood sample without buffy coat was centrifuged at 1,200 × *g* to obtain plasma. MNCs were washed and resuspended in HBSS with 10% autologous plasma to make a final concentration of 100,000 cells/mL for ROS production.

### *Measurement of ROS Generation*

Heparinized venous blood samples were diluted in HBSS at a dilution of 1:9 and kept at room temperature. One-milliliter samples of diluted blood were pipetted into flat-bottom plastic tubes and incubated at 37°C in a Chronolog (Haverstown, PA) lumi-aggregometer for 3 minutes. Luminol (final concentration, 300 μmol/L) was then added and free radical generation was induced by fMLP (final concentration, 20 μmol/L). Chemiluminescence was recorded for 15 minutes (a protracted record after 15 minutes did not alter the relative amounts of chemiluminescence produced by various blood samples). Our method, developed independently, is similar to that published by Tosi and Hamedani.[12] In this assay system, the release of superoxide radical as measured by chemiluminescence has been shown to be linearly correlated with that measured by the ferricytochrome C method.[12] We further established that, in our assay system, there is a dose-dependent inhibition of chemiluminescence by superoxide dismutase and catalase, as well as diphenylene iodonium (DPI; data not shown), a specific inhibitor of NADPH oxidase, the enzyme responsible for the production of superoxide radicals. The specific inhibitory effect of DPI on NADPH oxidase has been established by Hancock and Jones.[13] Our assay system is exquisitely sensitive to DPI-induced inhibition at nanomolar concentrations.

### *Cortisol Measurement*

Cortisol measurements in serum were performed using an immunochemiluminometric (ICMA) kit obtained from Nichols Institute (San Jaun Capistrino, CA).

### *Statistical Analysis*

Chemiluminesence due to ROS generation is highly variable from one subject to another. Therefore, for comparison, all values were normalized to 100% for the baseline time point and the following values were expressed as percent of basal. Statistical analysis was performed using ANOVA for repeated measures.

## RESULTS

### *Plasma Cortisol (HC) Concentrations*

The mean (±SD) cortisol concentration before the injection of HC (100 mg) was 14.5 ± 2.33 μg/dL. The concentrations following injection were 434.7 ± 30.47 μg/dL at 30 minutes, 366.9 ± 53.55 μg/dL at 1 hour, 248.9 ± 36.38 μg/dL at 2 hours, 133.1 ± 18.82 μg/dL at 4 hours, 69.4 ± 10.16 μg/dL at 8 hours, and 11.4 ± 2.25 μg/dL at 24 hours (Fig 1).

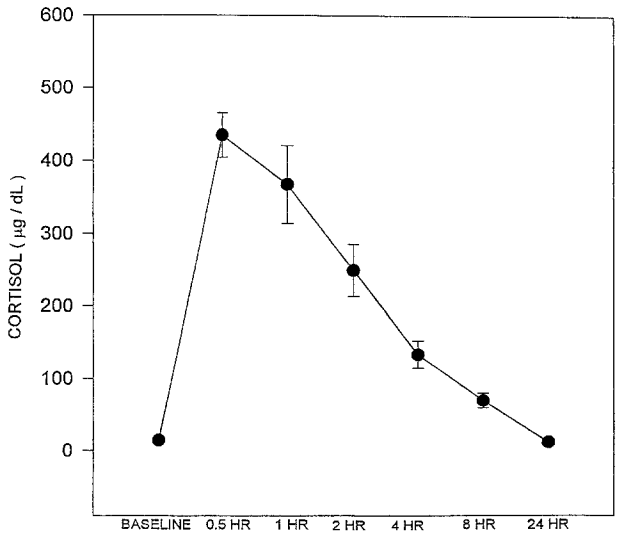


**Fig 1. Serum cortisol concentrations following intravenous injection of HC (100 mg). Note that the concentration of cortisol at 24 hours is similar to baseline. Values expressed at each time point are the mean ± SD.**

Following 300 mg intravenous HC, the serum concentration of cortisol was markedly higher, as expected. The concentration of cortisol at 24 hours was consistently lower than that before the 300 mg HC injection and that at 24 hours after the 100-mg injection of HC. This was presumably due to the suppression of endogenous cortisol secretion 24 hours after the injection of 300 mg HC. Thus, during the 8-hour period following injection of HC, even at the lower dose (100 mg) of HC, the serum concentration of HC was significantly greater than the physiological concentrations of this corticosteroid.

### *Effect of HC on ROS Generation by MNCs*

*Effect of 100 mg HC.* The chemiluminescence provided by ROS generated by MNCs at 0 minute was highly variable between subjects (range, 45 to 78 mV). There was a marked decrease in each subject's ROS generation by MNCs within 1 hour by at least 20%.

There was a significant decrease in MNC-generated ROS following HC. Relative to normalized basal values of 100.00% ± 0.0%, ROS generation was 54.6% ± 14.3% at 30 minutes, 30.7% ± 6.76% at 1 hour, 34.5% ± 5.43% at 2 hours, 50.0% ± 4.43% at 4 hours, 85.19% ± 1.14% at 8 hours, and 88.7% ± 3.25% at 24 hours. In brief, following the dose of 100 mg HC, ROS generation decreased significantly at 1 hour (nadir, 30% of basal; $P < .01$) and gradually recovered to nearly basal level at 8 hours (Fig 2).

*Effect of 300 mg HC.* Again, there was a significant decrease in MNC-generated ROS following HC. Relative to normalized basal values of 100.00% ± 0.0%, ROS generation was 73.5% ± 26.7% at 30 minutes, 30.6% ± 6.76% at 1 hour, 8.30% ± 3.69% at 2 hours, 26.99% ± 5.31% at 4 hours, 20.33% ± 8.16% at 8 hours, and 48.26% ± 12.71% at 24 hours. Therefore, following 300 mg HC, fMLP-induced ROS genera-

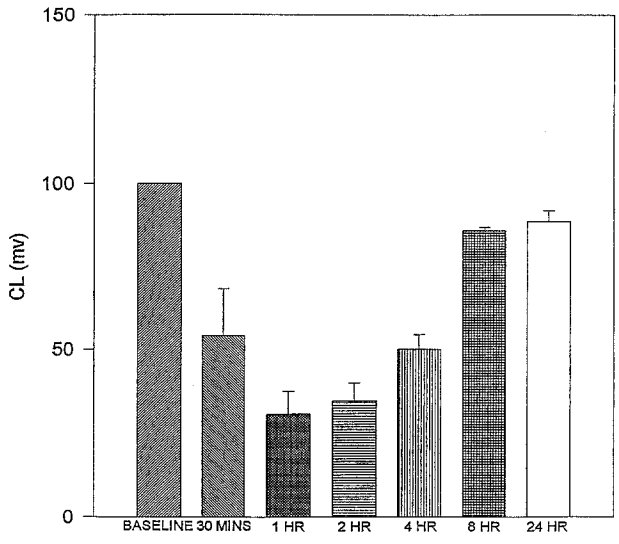


**Fig 2. Inhibitory effect of 100 mg HC on fMLP-induced ROS generation by MNCs. A significant inhibitory effect is observed at 30 minutes, with a nadir at 1 hour. Thereafter, the effect diminishes, with restoration of ROS generation to 85% of basal at 8 hours. Values expressed at each time point are the mean ± SD.**

tion decreased significantly by 27% ($P < .01$) at 0.5 hours and reached a nadir at 2 hours (8% of basal, $P < .001$); thereafter, it gradually recovered, but was still below baseline at 24 hours (Fig 3). Using Friedman's test for ANOVA for nonparametric data, the ROS readings at all time points were shown to be significantly lower than those before HC injection.

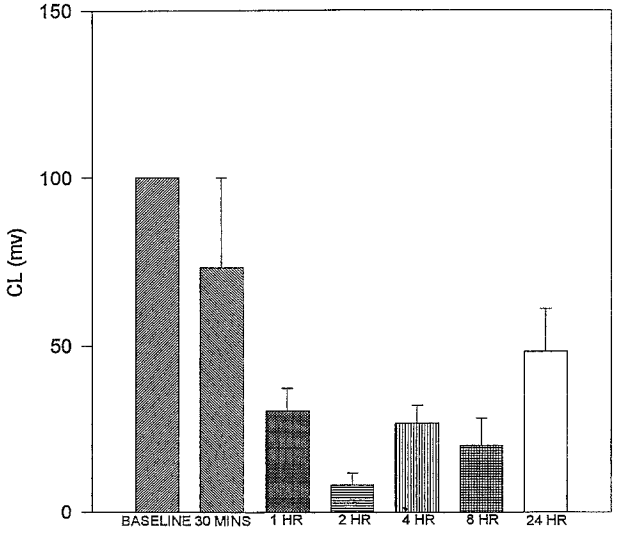


**Fig 3. Inhibitory effect of 300 mg HC on fMLP-induced ROS generation by MNCs. A significant inhibitory effect is observed at 1 hour, with a nadir at 2 hours. The generation of ROS inhibition continued to be significant even at 24 hours. Values expressed at each time point are the mean ± SD.**

## DISCUSSION

These data clearly demonstrate that ROS generation by MNCs is inhibited acutely following a single intravenous injection of HC at doses of 100 and 300 mg, respectively. The onset of this effect was observed at 30 minutes in each of the subjects studied, and the effect persisted for at least 24 hours, with a nadir of ROS generation at 2 hours for the 300-mg dose. The nadir of ROS generation following 100 mg was at 1 hour, following which ROS generation gradually increased to 85% of basal at 8 hours. The recovery of this profound inhibitory effect occurs from 4 hours onwards, but is gradual. Clearly, if patients are treated with higher doses or are given repeated doses of HC or similar corticosteroids, there would be further cumulative effects of the drug on ROS generation, which may continue to be inhibited for protracted periods during the course of therapy with these drugs. A dose of 60 mg of prednisolone is equivalent to 300 mg of HC and is commonly used in conditions characterized by immune inflammation (like systemic lupus erythrematosis), while a dose of 1,000 mg of methylprednisolone (equivalent to 5,000 mg HC) is used during transplant rejection therapy.

This inhibitory effect of glucocorticoids on ROS generation would be of use in terms of suppressing inflammatory responses. However, this effect could compromise the body's defense mechanisms, which may alter its ability to combat infections. It is well known that corticosteroid-treated patients have problems with handling infections and are often victims of overwhelming infections. This is probably the result of leukocyte suppression, including ROS generation, which is responsible for bacterial killing.

The mechanism underlying the inhibitory effect of corticosteroids on ROS generation is not clear from our experiments. However, corticosteroids are known inhibitors of the enzyme phospholipase $A_2$, which is known to facilitate ROS generation.[14] Recent work has demonstrated that in certain cell lines, glucocorticoids exert their effect by inducing the expression of the protein IκB, which binds to NFκB, which, in turn, is involved in the inflammatory pathway induced by endotoxin and tumor necrosis factor-alpha (TNFα).[15,16] We have previously demonstrated that endotoxin induces TNFα[17] and increases ROS generation[18] in humans in vivo. Therefore, it is possible that glucocorticoids induce the inhibition of ROS generation by IκB-induced inhibition of NFκB. We are currently investigating this possibility.

In conclusion, a single dose of 100 or 300 mg of HC promptly inhibits ROS generation by MNCs. This effect has hitherto not been described in patients or subjects given corticosteroids. These observations are consistent with data obtained previously in vitro and also provide us with the duration and the intensity of this inhibitory effect following doses of HC that are commonly used in clinical medicine. These effects probably explain a significant component of the antiinflammatory effect of corticosteroids in vivo and also the side effects of corticosteroid therapy in terms of immunosuppression. Larger doses naturally would be expected to have more profound and more lasting effects.

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