Role of Antioxidant Steroids in the Course of Bacterial Infection: Structure-activity Relationship of Steroids Regarding Antioxidant and Scavenger Capacity

Ph.D. thesis

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I. INTRODUCTION

The increasing incidence of chronic diseases caused by unhealthy lifestyle is a great problem of civilized societies. The common pathogenic agent of these disorders is oxidative stress deriving from the increased production and impaired neutralization of reactive oxygen species. Although a number of antioxidant drugs and dietary supplements are available, the beneficial effect of these in the mentioned diseases is not established. Estradiol and some other steroid hormones produced in the adrenal gland, testicles and ovaries have been shown to have antioxidant properties.

Hormone treatment – apart from the supplementation necessary in states of hormone deficiency – is not an acceptable complementary therapy of chronic diseases due to the many, frequently unfavorable side-effects. However, by recognizing the relationship between the steroid structure and antioxidant activity, it would be possible to develop drugs that ameliorate the oxidative state without the adverse steroid effects.

Based on previous results of our research group I examined the *in vivo* antioxidant capacity of some

hormones, and the effect of tibolone – a synthetic steroid with estrogenic, progestagenic and androgenic properties – and its metabolites on superoxide production of granulocytes.

The mortality of severe bacterial sepsis is high even with modern antibiotic therapies. Severe sepsis and septic shock are often associated with corticosteroid insufficiency caused by deficient cortisol production, altered cortisol metabolism, tissue resistance to cortisol effects or endothelial dysfunction in the adrenal gland. The inadequate intracellular cortisol activity results in exaggerated inflammatory response or the imbalance of pro- and anti-inflammatory mediators. In this state small dose of glucocorticoid supplementation may be beneficial, and is even recommended in cases where fluid and vasopressor treatment is not enough to maintain hemodynamic stability. Other steroid hormones are not used with this indication in medical practice; however, estradiol and testosterone also have immunomodulant effects. These hormones directly act on many cell types of the immune system, and increase myeloperoxidase (MPO) activity of neutrophil granulocytes. The primary physiologic role of MPO during granulocyte activation is

the promotion of free radical production and the killing of phagocytosed bacteria, and besides this our research group has previously attributed some antioxidant activity to MPO. Indomethacin, the inhibitor of MPO is used in inflammatory diseases of joints and muscles. According to our previous results, indomethacin inhibits the superoxide decreasing effect of antioxidant steroids on isolated granulocytes. This also confirms our hypothesis that the antioxidant activity of steroid hormones takes effect at least in part through enhancing MPO activity.

II. AIMS

In order to further examine the effect of steroid hormones I aimed to answer the following questions:

1. How does tibolone, a drug used in postmenopausal hormone replacement therapy and its metabolites affect superoxide production of activated human neutrophil granulocytes?
2. Does the antioxidant property of cortisol and corticosterone shown on isolated granulocytes realize also in *in vivo* circumstances?

As a sequel to the previous research regarding MPO, indomethacin and steroids that increase MPO activity, the following questions were raised:

3. How do antioxidant steroid hormones, MPO and indomethacin affect the bactericidal function of isolated and infected human granulocytes?
4. How does indomethacin, estradiol and testosterone treatment influence inflammation and bacteremia in a rat sepsis model?

III. METHODS

The effect of tibolone and its metabolites on superoxide production

Neutrophil granulocytes isolated from the blood of healthy volunteers were incubated for two hours at 37°C with either of the following compounds: tibolone, 3α-hydroxytibolone, 3ß-hydroxytibolone, Δ^4-tibolone, 3α-sulfated-tibolone, 3α-17ß-disulfated-tibolone, 3ß-sulfated-tibolone, 3ß-17ß-disulfated-tibolone, and estradiol as a reference.

The superoxide production of cells after stimulation with fMLP was measured with photometry as the reduction of cytochrome c. Using the molar extinction coefficient of cytochrome and standardizing the output per 10^6 cells, results are given as nmol superoxide anion/ 10^6 cells. Results for different steroids were given as percentages of their controls.

The compounds were tested also on a cell free superoxide producing system, the xanthine – xanthine-oxidase reaction.

Statistical analysis was performed using General Linear Models and Dunnett's 't' test to compare each treatment versus control.

The examination of the scavenger capacity of cortisol and corticosterone

For the experiment 16 adult male Sprague-Dawley rats were used. Four groups were created: untreated control, lipid rich diet control, cortisol treatment and lipid rich diet, corticosterone treatment and lipid rich diet. Total scavenger capacity was measured using chemiluminometric assay from blood samples taken just before treatment and on the 28^{th} day. The relative luminescence was determined by comparing it to a blank control and results were given as relative light units (RLU). Lower RLU resembles higher scavenger capacity. The mean RLU results measured from samples of day 0 and day 28 were compared with paired sample two-tailed *t*-test (SPSS v.12).

The effect of indomethacin and certain steroid hormones on intracellular bacterial killing

Granulocytes were isolated from the blood of healthy volunteers and incubated for 2 hours at 37°C in the presence of either indomethacin, MPO, 17ß-estradiol or cortisol. The cells were then mixed with opsonized *E. coli* bacteria, and after staining with acridine orange intracellular bacterial killing was evaluated visually under UV microscope. Acridine orange stains only dead bacteria, which fluoresce in orange when excited by ultraviolet light. The number of dead bacteria was counted and results are given as the percentage of control.

T**he effect of indomethacin, estradiol, and testosterone after *P. multocida* infection in an animal model**

For the experiment adult male Wistar rats were used, and the following treatments were applied:

Ii: indomethacin treatment and infection with *P. multocida*

I0: indomethacin treatment

Mi: treatment with the solvent of indomethacin and infection

M0: treatment with the solvent of indomethacin

CT: castration, testosterone treatment, infection

CE: castration, estradiol treatment, infection

C0: castration, treatment with the solvent of hormones, infection

NT: testosterone treatment, infection

N0: treatment with the solvent of hormones, infection

On the 2nd (indomethacin treatment) or 3rd (hormonal treatments) day after inducing infection, blood count and serum inflammatory parameters were determined. From the liver and heart blood of the infected rats the infective agent was reisolated on blood agar.

To compare the laboratory results ANOVA and Kruskall-Wallis test and LSD post hoc test (Statistica software) were used.

IV. RESULTS

The effect of tibolone and its metabolites on superoxide production

The superoxide production of granulocytes treated with steroids in 10^{-9} M concentration did not change significantly. Using 10^{-8} M of steroids, estradiol (80.9±2.5%, $p<0.05$), 3β-sulfated-tibolone (83.3±4.7%, $p<0.05$) and 3β-17β-disulfated-tibolone (81.0±4.2%, $p<0.05$) decreased superoxide production significantly compared to control. At 10^{-7} M concentration more metabolites had this effect: estradiol (76.4±4.2%, $p<0.001$), 3β-hydroxytibolone (82.9±5.3%, $p<0.05$), 3α-sulfated-tibolone (81.1±4.4%, $p<0.05$), 3β-sulfated-tibolone (79.2±5.7%, $p<0.01$), 3β-17β-disulfated-tibolone (74.6±5.1%, $p<0.0001$). Other tibolone metabolites (tibolone, 3α-hydroxytibolone, Δ^4-tibolone, 3α-17β-disulfated-tibolone) did not decrease significantly the superoxide production of granulocytes in any of the used concentrations.

In the xanthine – xanthine-oxidase system hormones were examined only in 10^{-7} M concentration. Significant

decrease in superoxide production was shown after treatment with estradiol (67.4±1.0%, $p<0.05$), 3α-sulfated-tibolone (85.8±5.3%, $p<0.05$), 3α-17β-disulfated-tibolone (71.9±2.5%, $p<0.05$), 3β-sulfated-tibolone (73.9±5.0%, $p<0.05$) and 3β-17β-disulfated-tibolone (65.8±3.4%, $p<0.05$) compared to control (100%). The rest of the steroid metabolites did not lead to significant change in superoxide production in this system.

The effect of indomethacin and certain steroid hormones on intracellular bacterial killing

The bactericidal capacity of neutrophils did not change after indomethacin treatment compared to control (103.3±47.6%). On the other hand MPO, estradiol and cortisol variably ameliorated bacterial killing: estradiol was the most effective (157.5±47.4%), then MPO (127.8±39.9%), and cortisol the least (121.7±52.3%). Due to small sample numbers statistical analysis was not done, only tendencies could be shown.

The examination of the scavenger capacity of cortisol and corticosterone

In the untreated control group relative luminescence did not change during the 28 days of the experiment. In the group on a lipid rich diet but no other treatment relative light unit was higher on day 28 than on day 0, which assumes a worse scavenging capacity; however, this change was not significant. Decreased scavenging capacity is most likely caused by oxidative stress induced by lipid load. In the groups treated with cortisol or corticosterone RLU was significantly lower on day 28 than day 0 (0.076±0.037 *vs.* 0.11±0.021 and 0.084±0.066 *vs.* 0.172±0.052, respectively).

T**he effect of indomethacin, estradiol, and testosterone after *P. multocida* infection in an animal model**

Infected animals were weak and faint, mostly the ones treated with indomethacin (infected and not infected as well).

White blood cell (WBC) count was significantly higher in group Ii than in groups I0 and Mi. In groups I0 and Ii red blood cell, hemoglobin, hematocrit, and serum albumin levels were lower compared to groups M0 and Mi. CRP was significantly higher in group Mi than in other groups, and it was lowest in group Ii.

Animals with infection and hormone treatments had lower WBC count: WBC was lower in group NT (not castrated, testosterone treatment) than in group N0 (not castrated control), and in groups CT and CE than in the control C0. In group CT a slight anemia developed, and red blood cell, hemoglobin and hematocrit was significantly lower than in the not castrated groups (N0, NT). Platelet count was lowest in group CE, with a significant difference compared to groups NT and C0. Serum protein levels were not significantly different in the various treatment groups, but albumin concentration was lower in all of the groups compared to the uninfected control (M0). CRP was significantly lower in group CE compared to the other groups. In group CT CRP was lower than in group C0, but this did not reach significance.

P. multocida colonies from the liver and heart blood of rats showed that the greatest number of bacteria could be reisolated from the animals in group Ii, with confluent colonies in 11 samples, *vs.* 3 samples in group Mi. In group Ii none of the samples gave a negative culture, however, in three samples in group Mi no colonies were reisolated.

In group NT fewer bacteria were cultured than in the control N0. Among castrated groups, from CT more and from CE less pathogens could be reisolated than from group C0. Regarding groups with testosterone treatment, more bacteria were cultured from castrated animals (CT) than not castrated ones (NT). Among control groups (N0, C0) no such difference could be shown.

V. CONCLUSIONS

Our research group has shown previously that many natural steroid hormones inhibit the superoxide production of activated neutrophil granulocytes. The mechanism of this action is not completely known, but it is partly mediated by the activation of MPO and the subsequent negative-feedback inhibition of NADPH oxidase.

In our present work the antioxidant effect of cortisol, corticosterone and the synthetic tibolone was evaluated. After this we examined the effect of MPO, some MPO activator steroid hormones and the MPO inhibitor indomethacin on the bacterial killing activity of neutrophil granulocytes and the outcome of sepsis.

My main results are the following:

1. Estradiol and some tibolone metabolites – 3ß-sulfated-tibolone, 3ß-17ß-disulfated-tibolone, and in a higher concentration 3ß-hydroxytibolone and 3α-sulfated-tibolone – decrease the superoxide production of activated isolated human neutrophil granulocytes.

2. Estradiol and the sulfated metabolites of tibolone (3α-sulfated-tibolone, 3α-17ß-disulfated-tibolone, 3ß-sulfated-tibolone, 3ß-17ß-disulfated-tibolone) decrease the superoxide generation of the xanthine – xanthine-oxidase system. Based on these it is possible, that the beneficial effects of tibolone in menopause are partly due to its scavenging activity.

3. Total scavenger capacity of rats kept on a lipid rich diet is decreased. Long term cortisol and corticosterone treatment significantly improve scavenger capacity. These hormones are thus antioxidant in the given experimental circumstances. In view of this and the known antioxidant activity of other steroid hormones it is possible to examine the structural background of this effect.

4. Acute myeloperoxidase, estradiol and cortisol treatment improves, indomethacin treatment does not affect the bacterial killing capacity of isolated human neutrophil granulocytes.

5. Indomethacin treatment and castration weaken the immune response and clinical state of septic rats. Indomethacin presumably increases the mortality of bacterial infections.

6. On the other hand testosterone, and even more so estradiol is beneficial in septic rats. While the results are ambiguous with testosterone, regarding estradiol, it might be worth to examine its efficiency as adjuvant treatment of septic patients. Prior to this, however, further animal experiments with greater sample numbers are needed.

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