Blood C reactive protein (CRP) and fibrinogen concentrations during staphylococcal experimental infection in obese dogs

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SUMMARY

The aim of the present study was to investigate the interactions between obesity and blood concentrations of 2 positive acute phase proteins, C reactive protein (CRP) and fibrinogen, in dogs during acute experimental infection with Staphylococcus intermedius. Staphylococcus infection was induced by subcutaneous injection of 24 hour broth culture of S. intermedius (5x10^9 CFU) in 6 healthy male adult mongrel dogs fed with a hypercaloric high-fat diet for 90 days (obese dogs) and in 8 non obese dogs whereas 6 other non obese dogs served as negative controls and plasma CRP and fibrinogen concentrations were monitored for 7 and 14 days, respectively. The CRP and fibrinogen concentrations dramatically increased in non obese infected dogs since 24 hours after inoculation, remained highly elevated for 2-3 days, then declined, slowly in the case of CRP and more abruptly in the case of fibrinogen. Increases in fibrinogen concentrations were more marked and prolonged and changes in the plasma CRP concentrations earlier appeared (since 3 hours after inoculation) and tended to be more important in obese infected dogs than in normal infected dogs. These results confirm that CRP and fibrinogen are 2 major positive APPs in dogs and show that obesity promotes the APP synthesis.

Keywords: Dog, Staphylococcus intermedius, experimental infection, obesity, C reactive protein, fibrinogen, acute-phase response, inflammation.

RÉSUMÉ

Concentrations circulantes de la protéine C réactive et du fibrinogène lors d’infection staphylococcique expérimentale chez les chiens obèses

Cette étude a eu pour objectif d’analyser les interactions possibles entre l’obésité et les concentrations circulantes de 2 protéines positives de la phase aigüe de l’inflammation, la protéine C réactive (PCR) et le fibrinogène chez le chien lors d’une infection expérimentale aiguë par Staphylococcus intermedius. L’infection a été induite par injection sous-cutanée d’une culture de 24 heures de S. intermedius (5x10^9 CFU) chez 6 chiens mâles adultes en bonne santé nourris pendant 90 jours par une ration hypercalorique riche en graisses (chiens obèses) ainsi que chez 8 chiens non obèses, alors que 6 autres animaux non obèses ont servi de contrôles négatifs. Les concentrations plasmatiques de la PCR et du fibrinogène ont été suivies sur des périodes de, respectivement, 7 et 14 jours. Chez les chiens infectés non obèses, les concentrations de la PCR et du fibrinogène ont considérablement augmenté dès 24 heures après inoculation, sont restées très élevées pendant 2-3 jours puis ont diminué, lentement dans le cas de la PRC et plus rapidement dans le cas du fibrinogène. Les augmentations de la fibrinogénémie ont été plus marquées et plus prolongées chez les chiens obèses infectés et de même, les modifications des concentrations de la PRC sont apparues plus tôt (dès la 3ème heure) et ont tendu à être plus importantes. Ces résultats confirment que la PRC et le fibrinogène sont 2 protéines positives majeures de la phase aigüe de l’inflammation et que l’obésité promeut la synthèse de ces protéines.

Mots clés : Chien, Staphylococcus intermedius, infection expérimentale, obésité, Protéine C réactive, fibrinogène, phase aigüe de l’inflammation.

Introduction

Acute-phase response is an important, non specific systemic reaction of organism seen through local and general disorders, which affect homeoasis and may be caused by infection or tissue damage of various aetiology (trauma, surgery, neoplasia and immune disorders) [7]. This reaction is the result of activation of natural defence mechanisms which are non specific and provide the needed time and modulating signals for the activation of specific immune response. Inflammation is the first reaction seen in tissue damage, which can be caused by non infectious or infectious agents. Infection sometimes occurs without causing inflammation – for example in immune deficient individuals. All cases of tissue damage lead to increased production and release of pro-inflammatory cytokines and changes in microcirculation. Pro-inflammatory cytokines, together with nitric oxide and glucocorticoids trigger and modulate acute-phase reaction through activation of hepatocytes [7].

Several hours after onset of infection, the ratio between different proteins produced by liver, changes dramatically. Plasma concentration of some proteins increases and these proteins are called positive acute-phase proteins whereas plasma concentration of others decreases and these proteins are called negative acute-phase proteins [7, 11]. Main positive acute-phase proteins include C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin (Hp), which are produced by hepatocytes after cytokine stimulation [8]. Acute-phase proteins are important defence mechanism reducing or stopping the development of pathogen microorganisms. Some proteins play the role of opsonins and activate the complement system, while others help elimination of cell debris and neutralisation of oxide radicals and proteolytic enzymes [15].
Some species-specific differences exist, mainly between mammals and birds. In spite of the differences, acute-phase proteins in humans and domestic animals are divided into 3 groups: 1) acute-phase proteins that increase their plasma concentration by 50%, like ceruloplasmin and C3 component of complement; 2) acute-phase proteins that mark two to three fold increase in their plasma concentration like haptoglobin, fibrinogen, α-globulin, and LPS-binding protein; 3) acute-phase proteins that rapidly increase their concentration, 5-1000 times higher than normal values such as CRP, SAA [16].

Functions of many acute-phase proteins are still not fully understood. CRP has an important role in the non specific defence against pathogen bacteria, fungi and parasites, by acting as an opsonin and activating the complement system through the classical pathway. CRP also binds necrotic and apoptotic cells and cell fragments [2, 6], thus stimulating phagocytosis and some pro-inflammatory effects. CRP was found in all atheromatous plaques investigated ex vivo [34, 37] in which this protein binds oxidised low density lipoproteins [2] and could activate the complement system causing inflammation in plaques. Fibrinogen is another positive acute-phase protein. It plays an important role in blood coagulation, cellular interactions, inflammation and wound healing. Its functions are accomplished by fibrin formation and complementary interactions with specific sites. Some cells, proteases, enzyme inhibitors and coagulation factors possess such sites [22]. As a part of acute-phase response, fibrinogen is linked to adhesion and migration of neutrophils and activation of their defence functions [9, 13, 31]. Binding of fibrinogen to specific toll-like receptors on macrophage surface, induces production of chemokines [24] and degradation products of fibrin suppress superoxide production by neutrophils and lead to decrease in lymphocyte blastogenesis [5]. ImmunoLogic stress induces release of adrenal gland hormones [22], which trigger acute-phase response. During acute-phase response, plasma viscosity increases as a result of changes in total proteins and some protein fractions. Elevation of fibrinogen is one of the most dramatic changes, which leads to increased erythrocyte sedimentation rate during inflammation.

Various factors influence immunity. Energy balance is one of the most important and integrating factors of physiological processes in organism. Recently, obesity has become a major problem not only in humans, but also in cats and dogs kept as pets. It was found that obese dogs are more susceptible to cardiovascular diseases, diabetes, reproductive, gastrointestinal, joint and skin problems, allergies, tumours and have lower resistance to viral and bacterial infections. Skin bacterial infections are very common among obese dogs and are most often caused by Staphylococcus intermedius, which is a typical residential microorganism in dogs, but in some conditions (allergic skin reactions, seborrhoea) is the main causative of canine pyoderma. In general, obesity has a negative effect on acute-phase response during infection.

Consequently, the aim of the present research is to study the interactions between obesity and circulating concentrations of two positive acute-phase proteins, CRP and fibrinogen, in dogs with acute subcutaneous Staphylococcus intermedius infection.

### Material and Methods

**ANIMALS AND PROTOCOL DESIGN**

A total of 20 healthy, male, mongrel dogs, 4-7 year old, were used in this study. Dogs were kept in individual cages situated indoors with a providing constant room temperature (18-21°C) and humidity comprised between 50 and 60%. Only natural light was used. Dogs were taken to a walk twice daily. Stuff conducting the experiment gave special care and attention to dogs in order to minimize stress during blood sampling and other manipulations. After a one month adaptation period, dogs were divided into three groups: dogs of the group I (n = 6, obese infected group) were fed with a hypercaloric high-fat diet for 90 days to induce obesity and then they were infected with Staphylococcus intermedius, dogs of the group II (n = 8, non obese infected group) were only infected with Staphylococcus intermedius and the group III (n = 6, control group) consisted in healthy (non infected) non-obese dogs.

When it was required, dogs were infected by a subcutaneous injection of 24-hour broth culture of Staphylococcus intermedius (density: 1x10⁹ CFU/mL, 5 mL). Staphylococcus intermedius strain used in the experiment was isolated and serotyped at the Department of Microbiology, Epidemic and Parasitic Diseases using semiautomated system for the identification BD BBL Crystal Gram Positive ID System.

The dogs from the three groups were fed a standard maintenance diet (“Jumbo Dog”, Gallisman S. A., Bulgaria) containing extruded grain, vegetable protein, fat, dehydrated poultry meat, amino acids, edible chestnut extract (tannin), plant extracts, vitamins and trace elements, minerals and antioxidants. The analytical content of the food was: protein-17%, fats-8%, fiber-4%, vitamin D₃-3000 IU/kg, vitamin E-200 mg/kg, vitamin A-11000 IU/kg, Zn-35 mg/kg, Na-0.4%, Mg-50 mg/kg, Ca-0.95–1.3%, Cl-0.95%, Fe-1.3%, Cr-0.95%, Cu-9 mg/kg, Na-0.95–1.3%, Mg-50 mg/kg, Ca-0.95–1.3%, Cl-1.05%, Cu-9 mg/kg, humidity-9%. Specifically, a lard supplement (10 g/kg body weight) was daily included in the diets given to dogs of the group I during 90 days.

Blood samples were collected by puncture of vena cephalica into sterile tubes with heparin as anticoagulant immediately before infection (hour 0), 3 hours after and on days 1, 2, 3, 7 and 14 (only for the fibrinogen determination) after the infection. After centrifugation (1500g, 15 minutes, room temperature), plasma were carefully harvested and stored at -20°C until assayed.

The experimental protocol was approved by the Ethic Committee of Trakia University.
**BIOCHEMICAL ANALYSES**

CRP (mg/L) was measured by an immune-enzyme method (Phase CRP – Canine Assay, Tridelta Development Ltd, Ireland). Fibrinogen (g/l) was measured using Hemostat Fibrinogen test set, (Human GMBH, Germany).

**STATISTICAL ANALYSIS**

Results were submitted to standard F- and t-tests (StatMost, version 2.5, DataMost Corporation). The data were presented as means ± standard deviation (SD). Differences were considered statistically significant at $P < 0.05$ level.

**Results**

In group I, the initial mean body weight was 12.9 ± 1.43 kg and increased to 16.5 ± 1.67 kg after 90 days of hypercaloric high-fat diet ($P < 0.01$) whereas in the 2 other groups (non obese dogs), the body weights remained constant for the whole experimental period. In the groups II and III, the initial body weights were 13.65 ± 3.31 kg and 12.43 ± 1.4 kg, respectively and the parameter did not change significantly after the 90 days period (13.83 ± 2.3 kg and 12.98 ± 1.7 kg in groups II and III, respectively). The mean body weight in group I at the end of the 90 day period was significantly higher compared to values observed in the 2 other groups ($P < 0.01$).

As shown in Table I, the plasma CRP concentrations were remained undetectable in healthy controls for the whole experimental period and they have dramatically increased since the first day after *Staphylococcus* infection in the 2 infected groups (obese and non obese dogs) (compared to 0 hour: $P < 0.001$) and remained significantly elevated until the 7th day compared to the initial values ($P < 0.001$ for the group I, $P < 0.01$ for the group II). Between the 1st and the 7th days post infection, the CRP concentrations recorded in the groups I and II were also significantly higher compared to the control values ($P < 0.001$). Furthermore, the increase in the CRP concentrations was detected 3 hours after infection but changes observed at this time point were statistically significant compared to the initial and control values ($P < 0.01$) only for the obese infected dogs. Although the CRP concentrations appeared slightly more elevated in the group I than in the group II, differences between the 2 groups of infected dogs were not statistically significant except at the 3rd hour after infection ($P < 0.01$).

The circulating fibrinogen concentrations were reported in Table II. In non obese infected dogs, this parameter gradually increased according to time (24h vs. 0h: $P < 0.001$), peaked on day 2 (48h vs. 0h: $P < 0.001$ and 48h vs.24h: $P < 0.05$).

<table>
<thead>
<tr>
<th>Group I (n = 6)</th>
<th>Group II (n = 8)</th>
<th>Group III (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hour 0.10 ± 0.00Å</td>
<td>0.10 ± 0.00Å</td>
<td>0.10 ± 0.00</td>
</tr>
<tr>
<td>3 hour 42.75 ± 25.05bbB</td>
<td>3.06 ± 4.40aaA</td>
<td>0.10 ± 0.00a</td>
</tr>
<tr>
<td>1 day 81.83 ± 0.4bcC</td>
<td>78.80 ± 4.38bbB</td>
<td>0.10 ± 0.00a</td>
</tr>
<tr>
<td>2 days 79.50 ± 3.88bcC</td>
<td>74.00 ± 9.80bbB</td>
<td>0.10 ± 0.00a</td>
</tr>
<tr>
<td>3 days 78.66 ± 3.80bcC</td>
<td>62.80 ± 19.38bbB</td>
<td>0.10 ± 0.00a</td>
</tr>
<tr>
<td>7 days 59.83 ± 24.60bcC</td>
<td>53.75 ± 29.24bbB</td>
<td>0.10 ± 0.00a</td>
</tr>
</tbody>
</table>

Different superscripts a,b in the same row indicate significant differences ($P < 0.01$ or more) between groups for a given time point.
Different superscripts A,B,C in the same column indicate significant differences ($P < 0.01$ or more) according to time after infection for a given group.

<table>
<thead>
<tr>
<th>Group I (n = 6)</th>
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<th>Group III (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hour 1.34 ± 0.20Å</td>
<td>1.68 ± 0.39Å</td>
<td>1.11 ± 0.03</td>
</tr>
<tr>
<td>3 hour 1.55 ± 0.44Å</td>
<td>1.65 ± 0.34Å</td>
<td>1.32 ± 0.24</td>
</tr>
<tr>
<td>1 day 3.06 ± 0.79bcC</td>
<td>3.78 ± 0.73bcD</td>
<td>2.82 ± 0.04a</td>
</tr>
<tr>
<td>2 days 6.17 ± 0.89abD</td>
<td>4.72 ± 0.80abD</td>
<td>1.77 ± 0.46a</td>
</tr>
<tr>
<td>3 days 3.30 ± 0.45bcC</td>
<td>4.12 ± 0.95bcC</td>
<td>2.84 ± 0.17a</td>
</tr>
<tr>
<td>7 days 3.99 ± 0.80bcC</td>
<td>2.60 ± 0.38abB</td>
<td>2.98 ± 0.31a</td>
</tr>
<tr>
<td>14 days 2.19 ± 0.67abB</td>
<td>1.60 ± 0.36åB</td>
<td>2.67 ± 0.12b</td>
</tr>
</tbody>
</table>

Different superscripts a,b,c in the same row indicate significant differences ($P < 0.05$ or more) between groups for a given time point.
Different superscripts A,B,C,D in the same column indicate significant differences ($P < 0.01$ or more) according to time after infection for a given group.

**Table I:** Variations of the plasma C reactive protein (CRP) concentrations (mg/L) according to time after a subcutaneous injection (5 mL) of *Staphylococcus intermedius* (24-hour broth culture, 5x10^9 CFU) in obese dogs (group I, n = 6) or in not obese dogs (group II, n = 8) compared to non obese, non infected dogs (group III, n = 6). Results are expressed as mean ± standard deviation.

**Table II:** Variations of the plasma fibrinogen concentrations (g/L) according to time after a subcutaneous injection (5 mL) of *Staphylococcus intermedius* (24-hour broth culture, 5x10^9 CFU) in obese dogs (group I, n = 6) or in not obese dogs (group II, n = 8) compared to non obese, non infected dogs (group III, n = 6). Results are expressed as mean ± standard deviation.
and then slowly declined (72h vs. 0h and 7 day vs. 48h or 72h: \( P < 0.001 \), 7 day vs. 0h: \( P < 0.01 \)) for reaching baseline values on day 14. Although the fibrinogen concentrations have moderately but not significantly fluctuated according to time in the non obese control dogs, it was observed that this parameter was significantly higher in infected dogs than in controls between the 1st and the 3rd days post infection (\( P < 0.05 \) on 24h and 72h, \( P < 0.001 \) on 48h) whereas on day 14, it was significantly lowered (\( P < 0.001 \)). In obese and infected dogs, the fibrinogen concentrations also markedly increased compared to the baseline values since the 24th hour (\( P < 0.01 \)) after *Staphylococcus* experimental infection, also peaked on day 2 (48h vs. 0h, 24h and 72h: \( P < 0.001 \)) and remained significantly elevated until the day 14 (0h vs. 72h and 7 days: \( P < 0.001 \) and 0h vs. 14 day: \( P < 0.05 \)). Differences with non obese control dogs were significant on days 2 (\( P < 0.001 \)), 3 and 7 (\( P < 0.05 \)). Moreover, the obese dogs exhibited higher fibrinogen concentrations than the non obese dogs, 2 (\( P < 0.05 \)) and 7 days (\( P < 0.01 \)) after the *Staphylococcus* inoculation.

**Discussion**

Changes in plasma concentrations of acute-phase proteins are part of acute-phase reaction. Though their physiological functions are not studied in details, they are known to play role in immune response regulation, inflammation, antimicrobial defence and reparative processes in impaired tissues [23]. Acute-phase proteins possess pro- and anti-inflammatory features, which are delicately balanced depending on various conditions [10]. Generally acute-phase response develops within several days and plays positive role as a part of innate defence mechanisms. Sometimes, in chronic inflammation acute-phase proteins are elevated for a longer period [18], and are involved in tissue damage and complications, such as the accumulation of non specific proteins in some tissues (amyloidosis) and cardio-vascular diseases [1, 30].

In the present study, it was observed that the experimental infection with *Staphylococcus intermedius* have induced marked increases in the circulating CRP and fibrinogen concentrations compared to initial values and to the control values in dogs. Furthermore, it was found that the CRP concentrations have remained very low and stable over the whole experimental period in the not infected dogs. Indeed, no circadian rhythm was evidenced for the blood CRP concentrations in healthy dogs [28] and various rhythms of sampling in a three week period have not significantly modified the CRP concentrations [19]. Consecutive blood sampling did not significantly alter the concentrations of acute-phase proteins in dogs [12, 19] and no change in CRP and ceruloplasmin concentrations was induced by glucocorticoid treatments [20]. In addition, no age related difference in blood acute-phase protein concentrations has been found in dogs [14]. Nevertheless, it was stated that the CRP concentrations greatly varied in healthy dogs according to the dog breeds and to some extent to the breeding conditions [28]. In contrast to the CRP concentrations in the not infected dogs, moderate fluctuations of the fibrinogen concentrations were observed in controls in the present study. These physiological changes may be attributed to the various actions of fibrinogen, notably in the blood coagulation [21]. Indeed, although changes in plasma fibrinogen concentrations during infection and inflammation have been well studied in dogs and other mammals and that a high fibrinogen concentration is a sign of systemic reaction to infection as fibrinogen is considered as a positive acute-phase protein [17], this protein is measured in routine veterinary practice mainly to indicate blood coagulation disorders but not to assess acute-phase response [21]. This is due probably to the fact that two- to four-fold increase in fibrinogen concentrations is less indicative for an acute phase reaction than multi-fold increase in concentrations of other proteins.

In many spontaneous and experimentally induced infectious diseases in dogs, it has been found that CRP is an acute-phase protein which rapidly and significantly increases [36]. In agreement with that, *Staphylococcus* infected obese and non obese dogs exhibited dramatically elevated plasma CRP concentrations since the 1st day after inoculation until the 7th day. However, it was not possible to establish the amplitude of the increase because the method employed here did not allow accurate quantification of low CRP concentrations in healthy dogs. In experimental *Staphylococcus aureus* infection, high circulating fibrinogen concentrations were found 24 hours after infection in dogs [27], reached maximal values 72 hours after and persisted elevated for 8 days [3, 4]. In the present study, it was also observed significant increases of the circulating fibrinogen concentrations in dogs infected with *Staphylococcus intermedius* since the 1st day after inoculation. Maximal concentrations were recorded on day 2 and then the APP concentrations gradually declined and reached baseline values or lower values in not obese dogs on day 14.

Nevertheless, it was observed that the circulating CRP concentrations has increased more rapidly and significantly, since 3 hours after bacteria inoculation, and appeared more elevated until the 7th day (although differences were not significant) in obese dogs than in non obese ones. In the same way, obese dogs exhibited higher blood fibrinogen concentrations than non obese ones, particularly on days 2 and 7 and the progressive decline in this parameter from the day 7 to the day 14 appeared to be delayed in fat animals. In obese animals, fat deposition increases and a large number of macrophages accumulate in the stroma-vascular fraction of intra-abdominal adipose tissue. It is known that fibrinogen stimulates the chemokine production by macrophages through activation of the macrophage inflammatory protein (MIP-1) and monocyte chemo-attractant protein (MCP-1) [5, 24], and that both macrophages and adipocytes possess superficial TLR-2 (Toll-like receptor-2) reacting to bacterial stimuli and leading to cell activation and increased production of IL-6 which is the main inductor of the liver CRP synthesis [1]. Consequently, the interleukin may be rapidly produced and in a great amount in obese dogs, leading to CRP over-expression. By elongating life span of fibrinogen in plasma, increased circulating concentrations of triglycerides and cholesterol [35] may contribute to the stimulating effect of fibrinogen on macrophages and indirectly to promote pro-inflammatory cytokines IL-1 and IL-6 synthesis. As one of the multiple effects of these cytokines is the dramatic change in hepatic protein synthesis and especially in fibrinogen production [21], the blood fibrinogen concentrations would be secondary greatly enhanced, creating an amplification pattern of the positive APP synthesis.
As a conclusion, these results confirm that *Staphylococcus intermedius* infection has induced rapid and important increases in both blood CRP and fibrinogen concentrations, confirming in this way that these inflammatory proteins are 2 positive APPs in dogs. In addition, these changes in circulating CRP and fibrinogen concentrations were more marked and prolonged in obese dogs, suggesting that adipocytes and lipid metabolism may indirectly interfere with the inflammatory response. These findings should be taken in consideration in clinical practice as markers of pathological processes and can be used to monitor the treatment efficiency.

**References**


