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Original Article

THE EFFECT OF PROPHYLACTIC INHIBITION OF INDUCIBLE NITRIC OXIDE SYNTHASE BY AMINOGUANIDINEON SERUM LEVELS OF SOME ADIPOCYTOKINES IN PRISTANE-INDUCED ARTHRITISIN RATS

SHATHA H. ALI[1], ALI A. KASIM[1,2]

[1]Department of Clinical Laboratory Sciences, College of Pharmacy, University of Baghdad, Baghdad-Iraq
Email: ali_a_kasim@yahoo.com

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ABSTRACT

Objective: The aim of the present study was to evaluate the effect of prophylactic inhibition of inducible nitric oxide synthase (iNOS) by aminoguanidine (AG) on serum levels of some adipocytokines in pristane-induced arthritis in rats.

Methods: Forty white Albino rats were divided randomly into four groups; each group was composed of ten rats (five male and five female). Group I (AP) has received AG (100 mg/kg/day) i. p. for seven days, and then at day 8, has received single 150 µl pristane dose sc. at the base of rat's tail. Group II (PC) has only received single 150 µl pristane sc. at the base of rat's tail, at day 8 from the start of the experiment. Group III (AC) has only received AG (100 mg/kg/day)i. p. for seven days. Group VI (VC) has only received normal saline via i. p. injection for seven days. At the end of the experiment time, rats were sacrificed, and serum samples were obtained and used for the measurement of iNOS, rheumatoid factor (RF), c-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), interleukine-6 (IL-6), leptin and adiponectin levels, using the corresponding rat ELISA kits.

Results: Administration of pristane for the induction of RA has resulted in significant increase in all of the measured parameters in PC group as compared to VC and AC groups, except for iNOS where the increase was significant as compared to AC group only. Serum levels of RF, CRP and IL-6 in AP group have showed to be significantly elevated as compared to VC and AC groups. Administration of AG has resulted in different levels of reduction in all of the measured parameters in AP group as compared to PC group. The reduction was statistically significant with regard to CRP and leptin.

Conclusion: prophylactic administration of the selective iNOS inhibitor AG, has resulted in a reduction in serum levels of the measured adipocytokines which may reflect a reduction in the severity of PIA.

Keywords: Adipocytokines, Aminoguanidine, iNOS, Pristane, Rheumatoid Arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, progressive, polyarthritic autoimmune disease associated with articular, and extra-articular or systemic effects [1]. It is one of the most common arthritides in western populations, with an estimated prevalence of 0.5-1.0% among adults. RA is more prevalent among women than men with a female preponderance of 2-3:1 [2, 3].

The perception of adipose tissue, as an organ that merely passively store excess fat and act as an energy store has changed. It is now considered as an endocrine organ that has an important role in regulating diverse physiological functions. This regulation is mediated by the action of biological mediators called "adipocytokines".

More than 50 different adipocytokines are currently recognized to be secreted from adipose tissue. These adipocytokines are implicated in the modulation of a range of physiological functions such as inflammation, glucose homeostasis, lipid metabolism, blood coagulation, angiogenesis, and blood pressure [4]. Adipose tissue produces and releases a variety of proinflammatory and anti-inflammatory factors, including the adipokines leptin, adiponectin, resistin, and visfatin, cytokines such as TNF-α, IL-6, and many others [5].

Nitric oxide (NO) is a free-radical messenger molecule that is produced from the amino acid L-arginine by the enzymatic action of NO synthase (NOS) [6]. Nitric oxide synthase (NOS) is available in both constitutive and inducible forms. Three different nitric oxide synthase (NOS) proteins have been identified, eNOS, iNOS and nNOS (endothelial, inducible and neuronal NOS, respectively) [7].

In autoimmune reactions and other forms of chronic inflammation, NO and NO-derived radicals can harm the host tissues [8]. Among its various biological actions, NO inhibits chondrocytes synthesis of collagen and proteoglycan, stimulates matrix metalloproteinases, and promotes vasodilation, which facilitates the influx of fluid and cells in to an inflammatory site. Furthermore, NO combines with reactive oxygen species, forming peroxynitrite, which promotes chondrocyte apoptosis [9].

Elevated NO production has been reported in RA patients [10, 11], and the increased urinary nitrite (metabolite of NO) concentrations have been shown to diminish after prednisolone treatment [12]. Elevated levels of NO were shown to correlate with several RA disease activity parameters [13]. Increased iNOS expression and NO production have also been detected in animal models of arthritis [14, 15], and the use of NOS inhibitors have been shown to have beneficial effects in experimentally induced arthritis [16, 17].

Aminoguanidine (AG) is a selective inhibitor of iNOS. It is over 50-fold more effective at inhibiting the enzymatic activity of iNOS than endothelial or neuronal isoforms of NOS [18].

Pristane-induced arthritis (PIA) in rats resembles the human disease such as in the development of symmetrical disease, the presence of serum rheumatoid factors, radiographic changes and chronicity [19]. The aim of the present study was to evaluate the effect of prophylactic iNOS inhibition by AG on serum levels of some adipocytokines in PIA in rats.

MATERIALS AND METHODS

Experimental animals

Forty white Albino rats of comparable age and sex, and weighing 150-200 gm were used in this study; they were obtained from and maintained in the Animal House of the Pharmacy College, University of Baghdad, in a climate-controlled environment. The animals were housed in polystyrene cages containing wood shavings and fed standard rodent chow and water ad libitum. The study was approved by the ethical committee at the Pharmacy College, University of Baghdad (Ethical approval number 5537; date 30th November 2014).

PIA induction and evaluation of arthritis

PIA was induced by a single subcutaneous (s. c.) injection of 150µl pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, St. Louis, Missouri, USA) at the base of rat's tail[19] at the age of 8–12 w. Arthritis development and severity were monitored in all four limbs every seven days by the perimeters of ankle and mid-paw, and a macroscopic scoring system. Briefly, the development of arthritis was monitored using a macroscopic scoring system for the four limbs ranging from 0-4 (1 = swelling and redness of one joint, 2 = two joints involved, 3 = more than two joints involved, and 4 = severe arthritis in the entire paw). The scores of the four paws were added, yielding a maximum total score of 16 for each rat [20].

Preparation of AG solution for injection

The daily dose of AG (Aminoguanidine HCl; Sigma-Aldrich, St. Louis, Missouri, USA) was prepared by dissolving the required amount in normal saline, and the dose was administered intraperitoneally (i. p.). AG dosage (100 mg/kg) was selected depending on the levels used effectively in experimental animal models for iNOS blockage [21-23].

Experimental protocol

Forty white Albino rats were enrolled in this experiment. The rats were divided randomly into four groups; each group was composed of ten rats, five of which were male and five were female. The study groups was treated as follows:

Group I (AP): has received AG treatment (100 mg/kg/day) i. p. for seven days, and then at day 8, has received single 150 µl pristane dose sc. at the base of rat's tail for the induction of RA.

Group II (PC): has received the pristane dose only (single 150 µl pristane sc. at the base of rat's tail for the induction of RA), at day 8 from the start of the experiment.

Group III (AC): has only received the AG treatment (100 mg/kg/day) i. p. for seven days.

Group VI (VC): has only received the solvent (normal saline) via i. p. injection for seven days.

Preparation of serum samples

After four weeks of last treatment, the animals have been anesthetized by ether, and then the blood was collected by cervical decapitation and transferred into a plain tube and left to clot for ten minutes before being centrifuged at 2,000 rpm for another 10 min. The sera were collected using rubber micropipette and divided into small aliquots in Eppendorf tubes and kept frozen till the time of analysis.

Biochemical analysis

Serum levels of iNOS, rheumatoid factor (RF), c-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), leptin, and adiponectin were measured by quantitative enzyme-linked immunosorbent assay (ELISA), using the corresponding readymade kits purchased from (Elabscience Biotechnology; China), and according to the manufacturer instructions.

Statistical analysis

Analysis of data was performed using GraphPad Prism software version 6.05 for Windows (GraphPad, San Diego, CA, USA). Values are presented as mean±SEM. The significance of difference among groups was tested by one-way analysis of variance (ANOVA), followed by Tukey's post hoc analysis to compensate for multiple testing. Homogeneity of variance was tested by, Brown-Forsythe's and Bartlett's Test. Statistical significance was considered whenever the P value was equal or less than 0.05.

RESULTS

Administration of pristane for the induction of RA has resulted in significant increase in all of the measured parameters in PC group as compared to VC and AC groups, except for iNOS where the increase was significant as compared to AC group only [(9.760±1.091 ng/ml vs. 5.100±0.6495 ng/ml for iNOS), (8.828±0.686kIU/lvs. 1.503±0.3833 kIU/land 1.786±0.3642kIU/l for RF), (22.15±1.532 mg/l vs. 7.06±0.760 mg/l and 9.01±1.238 mg/l for CRP), (55.00±4.874 pg/ml vs. 25.98±4.667 pg/ml and 34.79±5.346 pg/ml for TNF-α), (704.3±52.01 pg/ml vs. 175.5±30.71 pg/ml and 170.6±28.43 pg/ml for IL-6), (3.422±0.1923µg/l vs. 1.623±0.1857µg/land 1.998± 0.2108µg/l for leptin) and (47.09±3.464 ng/ml vs. 32.71±4.003 ng/ml and 33.26±3.822 ng/ml for adiponectin) respectively]. In AG treatment group (AP group), serum levels of RF, CRP and IL-6 have showed to be significantly elevated as compared to VC and AC groups [(7.913±0.4628kIU/l vs. 1.503±0.3833kIU/land 1.786±0.3642kIU/l for RF), (15.88 mg/l±1.416 vs. 7.06±0.760 mg/l and 9.01±1.238 mg/l for CRP) and (676.0±58.15 pg/ml vs. 175.5±30.71 pg/ml and 170.6±28.43 pg/ml for IL-6) respectively], as shown in table1.

Administration of AG has resulted in different levels of reduction in all of the measured parameters in AP group as compared to PC group. The reduction was statistically significant with regard to CRP and leptin [(15.88±1.416 mg/l vs. 22.15±1532 mg/l for CRP) and (1.917±0.2208µg/l vs. 3.422±0.1923µg/l for leptin) respectively], as shown in table 1.

Table 1: Serum levels of the studied parameters

	VC *n=10*	AC *n=10*	PC *n=10*	AP *n=10*	ANOVA P-value
iNOS (ng/ml)	6.211±1.239	5.100±0.6495	9.760±1.091[b]	7.200±0.8868	0.0152
RF (kIU/l)	1.503±0.3833	1.786±0.3642	8.828±0.686[a,b]	7.913±0.4628[a,b]	<0.0001
CRP (mg/l)	7.06±0.760	9.01±1.238	22.15±1.532[a,b,c]	15.88±1.416[a,b]	<0.0001
TNF-α (pg/ml)	25.98±4.667	34.78±5.346	55.00±4.874[a,b]	40.11±3.402	<0.0009
IL-6 (pg/ml)	175.5±30.71	170.6±28.43	704.3±52.01[a,b]	676.0±58.15[a,b]	<0.0001
Leptin (µg/l)	1.623±0.1857	1.998±0.2108	3.422±0.1923[a,b,c]	1.917±0.2208	<0.0001
Adiponectin (ng/ml)	32.71±4.003	33.26±3.822	47.09±3.464[a,b]	44.27±2.356	0.0082

Data are expressed as mean±SEM, VC= Vehicle control group, AC= Aminoguanidine control group, PC= Pristanecontrol group, AP= Treatment group (Aminoguanidine+Pristane), iNOS: Inducible Nitric Oxide Synthase, RF: Rheumatoid Factor, CRP: C - reactive protein, TNF-α: Tumor Necrosis Factor-alpha, IL-6: Interleukon-6. [a] = Significant difference as compared to VC group, [b]=Significant difference as compared to AC group, [c]= Significant difference as compared to AP group. A significant difference between groups is considered using ANOVA test whenever (P<0.05).

DISCUSSION

The detailed mechanisms of RA pathogenesis are not totally understood, and hence, the factors responsible for the initiation and progression of this disease are the core subject of many studies. The articular and synovial adipose tissue represents an important entity of human joints, and local and systemic alteration in the synthesis, release, and receptor action of adipocytokines needs further study to elucidate the potential role of these adipocytokines in RA.

In the present study, the administration of pristane for the induction of AR has resulted in a significant elevation in serum levels of iNOS, RF, CRP, TNF-α, IL-6, leptin and adiponectin, in PC group as compared to VC group (table1).

In addition to macrophages where it is primarily identified, iNOS expression can be induced almost in any cell or tissue, as long as the appropriate agents have induced its synthesis [24]. At the synovium of the inflamed joint macrophages, chondrocytes, osteoclasts and

osteoblasts express iNOS [25]. In addition, the inflamed synovium is invaded by T lymphocytes, B lymphocytes, neutrophils, monocytes, and dendritic cells [26] which also participate in iNOS expression.

In the present study, the administration of AG a selective iNOS inhibitor has resulted in small, non-significant decrease in iNOS levels in AC group as compared to VC group. This is very expected since the enzyme is usually not expressed in the normal physiological conditions. Nevertheless, AG treatment has resulted in a profound reduction (still statistically non-significant), in iNOS levels in AP group as compared to PC group. iNOS expression (and action) is controlled by large and different regulatory mechanisms[27, 28]. Some of these regulators are bone derived [29, 30]. On the other hand, adipose tissue has a role in inflammatory pathways, through the secretion of many cytokines that alter the expression of iNOS [31-34]. TNF-α has been found to upregulate iNOS transcription in synovial fibroblasts, articular chondrocytes and osteoblasts cultured from RA patients [10]. IL-6 also has been found to increase iNOS expression [35]. In human and murine chondrocytes, leptin synergizes with IL-1β and IFNγ for the activation of iNOS [36-38]. In cultured chondrocytes, adiponectin increases the secretion and activity of many proinflammatory mediators, including iNOS [39]. Thus, elevated serum levels of TNF-α, IL-6, leptin and adiponectin, reported in the present study may explain, at least in part, why AG has failed to bring down serum iNOS levels in the treatment group AP to that of VC group.

Rheumatoid factors (RFs) are typically produced during secondary immune responses against infections or immunizations [40]. RFs are detected in 60–80% of RA patients [41]. High titer RF is associated with radiologic erosion, extra-articular manifestations and thus, poorer outcomes [41-44]. RF has proven to be the most useful disease marker of RA, as included in the American College of Rheumatology classification criteria for RA [45].

The animal model that has been used in the present study, PIA rat model, resembles the human disease in the development of symmetrical disease, chronicity, radiographic changes and presence of serum RF [19].

C-reactive protein (CRP) is an acute-phase reactant that is commonly used in the diagnosis and follow-up of disease activity in rheumatology clinics. Even though it is called acute phase reactants, CRP rises in both acute and chronic inflammatory conditions [46].

In the present study, the administration of pristane has resulted in significant increase in CRP levels in both PC and AP groups as compared to VC and AC groups. This is greatly expected since pristane induces both cellular and humoral responses during the course of RA induction [47].

The synthesis of CRP is regulated primarily by interleukin-6 (IL-6), which in turn is controlled by other inflammatory cytokines, for instance, TNF-α and IL-1[48-50]. This occurs in accordance with the results of the present study regarding IL-6 and TNF-α (table1), which has showed a significant increase in IL-6 and TNF-α in pristane-treated groups.

Adipokines have been noted as new mediators of inflammatory processes [51, 52]. Many studies have demonstrated an elevated serum level of many adipokines in patients with RA, and an association between the levels of these adipokines and CRP [53-55]. In the present study serum levels of both leptin and adiponectin (table 1) have been shown to be elevated in pristane-treated groups. Thus, may further contribute to the elevated CRP levels.

Tumor necrosis factor-alpha **(**TNF-α), exhibits both the proinflammatory and immunoregulatory properties of cytokines. It is produced mainly by monocytes and macrophages, but also by B-cells, T-cells fibroblasts and adipocytes [56].

In the present study, the administration of pristane has resulted in significant increase in serum TNF-α level in PC group, as compared to VC and AC groups (table1). This result agrees with the findings of many clinical and experimental studies which have reported high synovial and serum levels of TNF-α in RA, and that TNF-α plays an important role in inflammation and joint destruction that are hallmarks of the disease [57-61].

TNF-α is one of the key cytokine molecules that causes inflammation in RA, by both direct [58], and indirect action through stimulating the release of other proinflammatory cytokines such as IL-6 [62-64]. Furthermore, TNF-α and leptin control the expression of each other. In one hand, TNF-α enhances the expression of leptin and its receptors [65]. On the other hand, leptin enhances TNF-α expression [66-68]. Thus, TNF-α may be an important contributor to the elevated serum levels of IL-6 and leptin, reported in the present study.

Interlekin-6 (IL-6) plays a role in adaptive immune responses involved in the pathogenesis of RA. In addition, this cytokine is responsible for mediating many of the systemic manifestations of RA [50].

In the present study, the administration of pristane has resulted in significant increase in serum IL-6 levels in PC and AP groups as compared to VC and AC groups (table1). This result agrees with the clinical findings that have reported an excess production of IL-6 in the synovial fluid and blood of RA patients and correlates with the disease activity and joint destruction [69, 70].

IL-6 induces the acute-phase response, particularly the development of CRP [71], which suggests that IL-6 contributes to the elevated serum levels of CRP reported in the present study.

Leptin is mainly produced by white adipose tissue and the circulating levels of leptin correlate positively with the amount of adipose tissue and body mass index (BMI) [72]. However, leptin synthesis is also regulated by the action of inflammatory mediators [73].

In the present study, the administration of pristane has resulted in significant increase in leptin levels in PC group as compared to VC and AC group (table 1). This result is in accordance with many studies that have reported elevated leptin levels in patients with RA and that serum leptin correlates with synovial fluid: serum leptin ratio and disease duration and parameters of RA activity [74-76]. Moreover, leptin has been found to correlate with CRP level in RA patients, which indicates that it may act as a proinflammatory cytokine in RA [53]. Generally, leptin behaves as proinflammatory cytokine due to its synergistic role with IFN-γ and IL-1β on iNOS [36-38].

The actions of leptin in RA are not only targeted to articular tissues, this adipokine also exerts direct modulatory effects on activation, proliferation, maturation and production of inflammatory mediators in a variety of immune cells, it activates monocyte/macrophage cells and increases production of the pro-inflammatory cytokines (TNF-α and IL-6) and reactive oxygen species (ROS) [77].

Some studies have reported conflicting data on serum levels of leptin in RA patients. Leptin levels were reported in some studies to be similar [78, 79], or even lower [80] in RA patients as compared with healthy controls. The discrepancies in the measured leptin levels may be attributed to the fact that, leptin levels are influenced by many factors such as body fat content, BMI, treatment, and disease activity and progression [81].

Adiponectin is produced mainly by white adipose tissue. It has structural homology with collagens VIII and X and complements factor C1q and the proinflammatory cytokine TNF-α, and it circulates in the blood in relatively large amounts in different molecular forms [82, 83].

Adiponectin concentration is decreased in diseases associated with low-grade inflammation [84, 85]. Moreover, its production is suppressed by inflammatory cytokines such as TNF-α and IL-6 [34]. On the other hand, adiponectin exerts a variety of anti-inflammatory effects on metabolic pathways and vasculature [86]. However, adipokines are elevated in RA and other classical inflammatory diseases [87-89].

Despite the discrepancy, adiponectin may have proinflammatory rather than anti-inflammatory properties in RA. Adiponectin shares extensive sequence homologies with the complement factor C1q and the pro-inflammatory cytokine TNF-α [82, 83]. Furthermore, adiponectin has demonstrated to induce the production of IL-6 and pro-MMP1 [89-91], key mediators of destructive arthritis.

In the present study, the administration of pristane has resulted in significant increase in serum adiponectin levels in PC group as compared to VC and AC groups (table1). This result is in total agreement with the findings of many studies that have reported that the amount of adiponectin is increased in the synovial fluid [92], and serum[93]of patients with RA, with higher levels in erosive versus mild RA [93-95], and in chronic versus early RA [96].

Articular adipose tissues may be the main source of adiponectin in RA, yet, serum adiponectin level has been reported to be higher than in synovial fluid in RA[93], suggesting that other systemic sources are more prominent than articular adipose tissues. Furthermore, the negative association of glomerular filtration rate and serum adiponectin level has been reported [97], indicating that impaired renal clearance of adiponectin may lead to increased adiponectin level in inflammatory diseases as well.

Finally, in the present study the administration of the selective iNOS inhibitor AG, has resulted in a reduction in serum levels of RF, CRP, TNF-α, IL-6, leptin and adiponectin, in AP group as compared to PC group. The reduction was statistically significant with regard of CRP and leptin (table 1). This reduction may be attributed to the decrease in the severity of PIA by the administration of AG.

This study is rather limited in terms of statistical power because of the small sample size and further large-scale animal and clinical studies are needed to establish our findings. Also, further work is required to confirm whether the reported effects are confined to AG or the whole members of iNOS inhibitors family of agents.

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CONFLICT OF INTERESTS

Declared None

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