Decreased Gene Expressions of Insulin Signal Molecules in Canine Hyperadrenocorticism

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ABSTRACT. Hyperadrenocorticism (HAC) is a common endocrine disorder in dogs, in which excess glucocorticoid causes insulin resistance. Disturbance of insulin action may be caused by multiple factors, including transcriptional modulation of insulin signal molecules which lie downstream of insulin binding to insulin receptors. In this study, gene expressions of insulin signal molecules were examined using neutrophils of the HAC dogs (the untreated dogs and the dogs which had been treated with trilostane). Insulin receptor substrate (IRS)-1, IRS-2, phosphatidylinositol 3-kinase (PI3-K), protein kinase B/Akt kinase (Akt)-2 and protein kinase C (PKC)-lambda were analyzed in the HAC dogs and compared with those from normal dogs. The IRS-1 gene expressions decreased by 37% and 35% of the control dogs in the untreated and treated groups, respectively. The IRS-2 gene expressions decreased by 61% and 72%, the PI3-K gene expressions decreased by 47% and 55%, and the Akt-2 gene expressions decreased by 45% and 56% of the control dogs, similarly. Collectively, gene expressions of insulin signal molecules are suppressed in the HAC dogs, which may partially contribute to the induction of insulin resistance.

KEY WORDS: canine, hyperadrenocorticism, insulin signal molecules, neutrophils

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Hyperadrenocorticism (HAC) is an endocrine disease which shows hyper-glucocorticoids in humans and dogs. Physiologically, glucocorticoids are produced in adrenal cortex under the control of hypothalamic-pituitary-adrenal axis and modulate carbohydrate metabolism [1, 11]. Glucocorticoids are inducers of insulin resistance, which defined as impaired sensitivity to the effects of insulin on carbohydrate metabolism, so the hyper-glucocorticoid is a significant risk factor of diabetes mellitus (DM) also in dogs [31, 33]. In rodents, corticosterone, which is a rodents' major glucocorticoid, does antagonistic actions on insulin, and increased plasma glucose concentrations had been observed in the corticosterone-treated rodents [7, 24, 29, 32]. Insulin stimulates glucose uptake via translocation of glucose transporter (GLUT)-4 from intracellular vesicles to cellular membranes in insulin-sensitive tissues (liver, skeletal muscle, adipose tissue, etc.). In human and rodent studies, excess glucocorticoids have shown to antagonize insulin action by inhibiting insulin-mediated glucose uptake and utilization by insulin-sensitive tissues [5, 16]. When

insulin binds to its receptors, the receptor becomes tyrosine-phosphorylated form and activates insulin receptor substrate (IRS) -1, 2, 3 and 4. Phosphorylated tyrosine on the IRS proteins binds to the p85 regulatory subunits of phosphatidylinositol 3' kinase (PI3-K), which is a heterodimer of a regulatory subunit (p85) and a catalytic subunit (p110) [9]. Recent reports indicate that the serine/threonine protein kinase protein kinase B (PKB; also known as Akt) and atypical protein kinase C (aPKC, PKC-lambda and -gamma) mediate the downstream events controlled by PI3-K and Akt-2, which are enriched in insulin-sensitive tissues and have been specifically implicated in the metabolic actions of the hormone [6, 17, 40]. The initial report suggesting that aPKC is required for insulin-stimulated glucose transport describes that inactive (dominant-negative) forms of these protein kinases cause inhibitory effects on insulin action in adipocytes and muscular cells [3, 19, 38, 44]. In our previous report, we measured glucose infusion rates (GIR) of HAC dogs by euglycemic-hyperinsulinemic glucose clamp (EHGC) method using an artificial pancreas apparatus [15]. EHGC method [12] is a gold standard for assessing insulin resistance in human skeletal muscles [18]. When GIR in the HAC dogs were compared with those in normal dogs, the HAC dogs showed lower GIR than normal ones, indicating that the HAC dogs are suffering from insulin resistance as well as human patients [15]. The mechanism of insulin resistance may include transcriptional, translational and activation modulations of insulin signal molecules. Currently, we focused on the transcriptional level, so measured gene expressions of IRS-1, IRS-2, PI3-K, Akt-2 and PKC-lambda

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Table 1. Profiles of the dogs

No.	Classification	Breeds	Age (years)	Gender	Cortisol concentration ($\mu\text{g/dl}$)		Adrenal glands sizes (mm)		Clinical signs
					pre ACTH stimulation	post ACTH stimulation	Right	Left	
1	Control	Beagle	3	Spayed	<1	14.1	4.6	4.9	no sign
2		Beagle	3	Spayed	<1	N.A.	5.6	4.4	no sign
3		Beagle	3	Castrated	1.26	14.3	N.D.	4.0	no sign
4		Beagle	2	Castrated	<1	11.7	N.D.	4.9	no sign
5		Beagle	2	Castrated	1.11	15.7	N.D.	3.6	no sign
6		Beagle	2	Spayed	1.02	16.7	N.D.	4.0	no sign
7		Beagle	6	Spayed	2.11	10.6	N.D.	N.D.	no sign
8		Beagle	6	Spayed	1.78	9.39	4.8	4.3	no sign
9	HAC (untreated)	Toy Poodle	6	Castrated	13.0	29.2	3.9	3.3	PU/PD, polyphagia, dermatologic problem
10		Portuguese Water Dog	8	Castrated	8.80	30.3	12.8	10.9	PU/PD, polyphagia, dermatologic problem
11		Tiny Poodle	13	Female	5.75	56.2	6.0	7.8	PU/PD, decreased activity, panting
12		Yorkshire Terrier	4	Spayed	16.5	46.4	5.5	7.3	PU/PD, dermatologic problem, decreased activity
13		Yorkshire Terrier	12	Male	9.82	23.7	7.5	7.2	PU/PD, polyphagia, decreased activity
14	HAC (treated)	Miniature Dachshund	14	Castrated	3.22 (8.60)	6.15 (37.1)	(7.6)	(5.4)	dermatologic problem, decreased activity, pendulous abdomen
15		Maltese	10	Female	2.21 (15.6)	5.90 (39.8)	(10.7)	(9.6)	PU/PD, polyphagia, dermatologic problem
16		Miniature Dachshund	12	Female	1.85 (9.07)	2.98 (23.4)	(6.6)	(5.9)	PU/PD, polyphagia, dermatologic problem
17		Miniature Dachshund	11	Castrated	1.95 (2.80)	5.62 (45.5)	(8.5)	(10.2)	polyphagia, dermatologic problem, pendulous abdomen
18		Mix	7	Spayed	3.70 (7.50)	8.60 (31.5)	(9.8)	(8.2)	PU/PD, polyphagia, dermatologic problem
19		Pug	11	Female	3.57 (5.75)	7.81 (53.3)	(7.0)	(7.6)	PU/PD, polyphagia, dermatologic problem

HAC, Hyperadrenocorticism; PU/PD, polyuria polydipsia; N.A., not applicable; N.D., not detectable.

In the treated HAC group, the cortisol concentrations and the sizes of adrenal glands noted in brackets are those at diagnosis (before treatments).

in the HAC dogs to reveal a possible mechanism of insulin resistance in HAC dogs. Demonstrating such mechanisms may lay out the pathophysiology of canine HAC and will be of help in exploring therapeutic strategy.

Eight beagles (3 castrated males and 5 spayed females, 2–6 years old) maintained in our laboratory were used as the control dogs. These dogs did not show any clinical signs and significant pathological variations in blood biochemistry (data not shown). Serum cortisol concentrations after ACTH administration were lower than $20 \mu\text{g/dl}$ in control dogs (Table 1). Eleven client-owned dogs suffering from HAC, visiting our veterinary medical teaching hospital, constituted the HAC group. Their profiles, serum cortisol concentrations and size of adrenal glands are shown in Table 1. All animals have not received glucocorticoids for medical or research purposes before the examinations. Diagnosis of HAC was confirmed on the basis of clinical signs and the ACTH stimulation test. Dogs were included in the study when at least 3 clinical signs of HAC (polyuria-polydipsia [PU/PD], polyphagia, dermatologic problems, decreased activity, panting and pendulous abdomen) were detected. A cortisol concentration of $>20 \mu\text{g/dl}$ in the sample obtained 1 hr after ACTH administration was considered abnormal and consistent with HAC. In addition, the adrenal glands were examined using ultrasonography. The HAC dogs were divided to two groups, such as the untreated dogs and the treated dogs which had been received medical treatments with trilostane. Serum cortisol concentrations after ACTH administration in the untreated dogs were higher than $20 \mu\text{g/dl}$. By contrast, those in the treated dogs were lower than $20 \mu\text{g/dl}$, while they had been higher than $20 \mu\text{g/dl}$ before

they received the treatment (Table 1). Approval for this work has been given by the Nippon Veterinary and Life Science University Animal Research Committee.

The blood samples were collected into EDTA-2K tubes. Neutrophils were separated from whole blood in accordance with a previous report [27]. The yield of neutrophils by this method is $>95\%$, both in our laboratory and in the previous report. Total RNA was extracted from the neutrophils using a commercial kit (QIAamp RNA Blood Mini, QIAGEN, Tokyo, Japan) and stored at -80°C until the assay. Quantitative real-time (qRT) PCR analysis was performed to compare the mRNA levels of insulin signal molecules in peripheral neutrophils. Total RNA samples were reverse-transcribed into cDNA using a QuantiTect Rev. Transcription Kit (QIAGEN). The primers were designed to the targeted genes using the sequences information (Table 2). The qRT-PCR reactions for each gene of interest were performed in triplicate. The reactions were carried out with a commercial kit (Perfect Real Time SYBR Premix Ex Taq II) using an Applied Biosystems 7300 Real Time PCR Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). The protocol was as follows, 94°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Following amplification, a melting curve analysis program was performed to verify the authenticity of the amplified products by their specific melting temperatures (Applied Biosystems). The resulting products were subjected to nucleotide sequencing to confirm their specificity. PCR amplification was carried out in $20 \mu\text{l}$ solutions containing $1 \mu\text{l}$ of template cDNA, $0.8 \mu\text{l}$ of each specific primer ($10 \mu\text{M}$), $10 \mu\text{l}$ of SYBR Premix Ex Taq, $0.4 \mu\text{l}$ ROX reference dyeII and $7.0 \mu\text{l}$ of distilled water.

Table 2. Primer sequences

Target	Expected size of the PCR product (bp)	Primer type	Sequence (5'-3')	GenBank Acc. No.
beta-actin	129	Forward	gccaacctgagaagatgact	AF021873
		Reverse	cccagagtcctatgacaataaccag	
IRS-1	81	Forward	acctgcgttcaaggaggctcg	XM543274
		Reverse	cggtagatgccaatcagggttc	
IRS-2	177	Forward	tggcagggtgaacctgaagc	XM542667
		Reverse	gaagaagaagctgtccgagtgg	
PI3-K	132	Forward	gcattaaccagacctcattcage	AB436616
		Reverse	gcgagtattgtcttcagtgtctc	
Akt-2	180	Forward	ctcgagtattgtcattcgag	NM001012340
		Reverse	acctggcaccgaggtgctg	
PKC-lambda	135	Forward	gctctgataaccggatcaa	XM535855
		Reverse	cctttgggtccttgttgaga	

After qRT-PCR amplification, absolute quantification was performed according to the method of Whelan *et al.* [47], by establishing a linear amplification curve from 10-fold serial dilutions of cloned and sequenced plasmid DNA containing target PCR products. The expression level of the beta-actin gene as a housekeeping gene was quantified by the same method, and the expression levels of the target genes were rectified by the expression level of the beta-actin gene. Each value of mRNA expression was denoted as the relative value of the control group.

Values were expressed as means $\pm$ standard errors of the mean (SEM). For statistical analyses among the groups, Kruskal-Wallis test was employed using Graph Pad Prism 5.2 (GraphPad Software, La Jolla, CA, U.S.A.).

The results of the gene expressions of insulin signal molecules, such as IRS-1, IRS-2, PI3-K, Akt-2 and PKC-lambda, examined in the 8 normal dogs, the 5 untreated HAC dogs and the 6 treated HAC dogs are shown in Fig. 1. IRS-1 mRNA expressions decreased by 37% in the untreated HAC dogs and 35% in the treated HAC dogs comparing to the control dogs, but the differences were not statistically significant. IRS-2 mRNA expressions decreased by 61% in the untreated HAC dogs and 72% in the treated HAC dogs comparing to the control dogs, and the difference in the treated HAC dogs was statistically significant ($P < 0.01$). PI3-K mRNA expressions decreased by 47% in the untreated HAC dogs and 55% in the treated HAC dogs comparing to the control dogs, and the difference in the treated HAC dogs was statistically significant ($P < 0.05$). Akt2 mRNA expressions decreased by 45% in the untreated HAC dogs and 56% in the treated HAC dogs comparing to the control dogs, and the difference in the treated HAC dogs was statistically significant ($P < 0.05$). PKC-lambda mRNA expressions did not change in both the untreated HAC dogs and the treated HAC dogs comparing to the control dogs.

As the mechanism of insulin resistance, there are multiple possibilities, such as decreased mRNAs (at transcriptional level), decreased proteins (at translational level), decreased phosphorylation (at activational level) of signal molecules and/or inhibition by other factors. In the present study, we focused on the transcriptional level, so we measured gene

expressions of the insulin signal molecules (IRS-1, IRS-2, PI3-K, Akt-2 and PKC-lambda) in HAC dogs and compared them to those in normal dogs. Currently, we used leukocytes for analysis, which are insulin-target cells [10, 14, 21, 28, 35], and insulin regulates glycolytic enzymes and GLUT translocations in leukocytes [23]. Actually, we have demonstrated that leukocytes reflect the gene expressions in other insulin-sensitive tissues, such as liver, skeletal muscle and abdominal fat, in a previous paper [25]. Total leukocytes were used in the previous study, but we used only neutrophils in this study, because glucocorticoids increase a number of peripheral neutrophils and decrease lymphocytes and then the proportion of leukocytes may change.

In the present study, IRS-1 and IRS-2 expressions were suppressed in the HAC dogs, as shown in Fig. 1. These molecules are ubiquitously expressed proteins, which are located in the head stream of insulin signal cascade [2, 30, 48, 49]. There are some reports which have described the effects of glucocorticoids on insulin signaling in rodents, i.e., decrease in phosphorylated IRS-1 and increase in phosphorylated IRS-2 levels *in vitro* [8, 36] and decrease in phosphorylated IRS-2 levels *in vivo* [4, 37, 39, 46]. In other reports, IRS-1/2 decreased in the liver and skeletal muscle of diabetic humans showing insulin resistance and *ob/ob* mice with type 2 diabetes [42, 43]. Furthermore, IRS-2 knocked out mice showed diabetic symptoms [20]. In the veterinary field, we have reported that IRS-2 proteins in the skeletal muscle, adipose tissue, liver and leukocytes of obese cats showed lower expression levels than those in normal cats [22, 26]. As for the cat, obesity is regarded as a risk factor of DM onset like human beings [34]. PI3-K and Akt-2 mRNA expressions in the HAC dogs significantly decreased. It has been reported that the mRNA expressions of Akt-2 were suppressed in cultured murine ovarian cells when dexamethasone was applied into the medium [45]. This report harmonizes the present result, in which the Akt-2 mRNA expressions decreased in the dogs with higher cortisol levels. In the current results, the insulin signal molecules had remained suppressed even after the HAC was treated. These findings square with a previous study, in which insulin sensitivity has been decreased in human HAC patients, and it has continued even after the

Fig. 1. Gene expressions of insulin signal molecules. Gene expressions of insulin signal molecules, such as IRS-1, IRS-2, PI3-K, Akt-2 and PKC-lambda, in neutrophils were analyzed in the control dogs (n=8), the untreated HAC dogs (n=5) and the treated HAC dogs (n=6). Values are expressed as means $\pm$ SEM (* P <0.05; ** P <0.01 vs. control, Kruskal-Wallis test).

glucocorticoid levels were recovered [13]. Collectively, gene expressions of insulin signal molecules, especially IRS-2, PI3-K and Akt-2, decreased in the HAC dogs, which may contribute to the induction of insulin resistance. In the current study, we have not analyzed GLUT-4 variations. The GIR of HAC dogs had been significantly lower than those of control dogs in our previous study [15], suggesting that GLUT-4 located in cell membranes had decreased in HAC dogs, although it is unclear if it decreased at a transcriptional level. A further study is necessary to clarify the transcriptional modulation of GLUT-4 in HAC dogs.

Limitation of this study lies in that the data have been collected from neutrophils. Variations of the insulin signal molecules in neutrophils might not always reflect that in other tissues, such as skeletal muscles, adipose tissues and liver. However, we have demonstrated that the insulin signal molecules mRNAs in neutrophils correlate with those in other tissues in cats previously [26], so we consider them

indirect measures to assess the condition of insulin signal molecules in other tissues. Recently, it has been reported that neutrophilic elastase is associated with insulin resistance [41]. Although it is unclear if the phenomenon is connected to insulin resistance in HAC dogs in this study, insulin has biological effects on neutrophils and it might be worth pursuing in further studies.

In conclusion, it was suggested that decrease in gene expressions of insulin signal molecules may be a mechanism of insulin resistance in HAC dogs, partially, at least. We focused on the transcription of insulin signal molecules, and as for the next challenge, we should analyze the protein amounts (translation) and the phosphorylation (activation) of these molecules to get the whole story. Moreover, to reveal detailed relationships of glucocorticoid and insulin signal molecules, the study should be extended to laboratory dogs receiving glucocorticoids experimentally or clinical patients receiving glucocorticoids for medical treatment (i.e.

autoimmune disease, inflammatory bowel disease, etc.). It is also necessary to compare the molecule actions and insulin resistance assessed by EHGC method in HAC dogs.

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