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Research Article

Altered Responses to Cold Environment in Urocortin 1 and Corticotropin-Releasing Factor Deficient Mice

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We examined core body temperature (CBT) of urocortin 1 (UCN1) and corticotropin releasing factor (CRF) knockout (KO) mice exposed to 4°C for 2 h. UCN1KO mice showed higher average CBT during cold exposure as compared to WT. The CBT of male and female WT mice dropped significantly to 34.1 ± 2.4 and 34.9 ± 3.1 C at 4°C, respectively. In contrast, the CBT of male and female UCN1KO mice dropped only slightly after 2 h at 4°C to 36.8 ± 0.7 and 38.1 ± 0.5 C, respectively. WT female and male UCN1KO mice showed significant acclimatization to cold; however, female UCN1KO mice did not show such a significant acclimatization. CRFKO mice showed a dramatic decline in CBT from 38.2 ± 0.4 at 22°C to 26.1 ± 9.8 at 4°C for 2 h. The CRF/UCN1 double KO (dKO) mice dropped their CBT to 32.5 ± 4.0 after 2 h exposure to 4°C. Dexamethasone treatment prevented the decline in CBT of the CRFKO and the dKO mice. Taken together, the data suggest a novel role for UCN1 in thermoregulation. The role of CRF is likely secondary to adrenal glucocorticoids, which have an important regulatory role on carbohydrate, fat, and protein metabolism.

1. Introduction

Corticotropin releasing factor (CRF) [1] plays an essential role in the physiological regulation of the hypothalamic-pituitary-adrenal (HPA) as CRF knockout (KO) mice have low concentrations of corticosterone and decreased response to stress [2]. CRF is also found in several regions of the central nervous system (CNS) where it functions as a neurotransmitter and regulates several aspects of behavior particularly in response to stressful stimuli [3].

The urocortins, UCN1 [4], UCN2 [5], and UCN3 [6], are a family of peptides, which were discovered by their sequence homology to CRF, sauvagine [7], and urotensin 1 [8]. These peptides bind and activate the CRF receptors, CRFR1 [9] and CRFR2 [10], with different affinities and potencies. CRF binds to CRFR1 with a higher affinity than to CRFR2, whereas UCN1, sauvagine, and urotensin 1 interact with both receptors with a relatively similar affinity [10]. In contrast, UCN2 and UCN3 have higher affinity for CRFR2 than for CRFR1 [5, 6]. Therefore, the actions of UCN1 can be mediated by both CRF receptors [11]. In this regard, it has been shown that UCN1 mRNA levels are upregulated in the Edinger-Westphal (EW) nucleus of mice following stress exposure [12]. UCN1-deficient mice were shown to have a normal corticosterone response to acute immobilization stress [13, 14]; however, they showed anxiety-like behavior and impaired inner ear physiology [13]. UCN1KO mice were also shown to have decreased corticosterone response to cold and impaired adaptation to repeated constraints [15].

A role for UCN1 in thermoregulation has been suggested from the finding that intracerebroventricular injection of UCN1 in rats significantly increased colonic temperature [16] and that a cyclooxygenase inhibitor reduced this effect of UCN1 [17].

In this study, we assessed the hypothesis that UCN1 may be important for the maintenance of core body temperature during exposure to cold environment and examined the acclimatization of the core body temperature to a 2 h cold exposure of mice deficient in UCN1, CRF, or both.

2. Materials and Methods

2.1. Animals. The UCN1KO mice, in the C57BL/6 background, were described by us previously [15]. The CRFKO

Table 1: Inner body temperature of UCN1KO mice at 4°C is higher than that of WT mice: statistical comparison of average rectal temperatures, taken every 10 min during 2 h (9 to 13 measurements for each mouse, n = 6-7 animals in each group) of exposure to 4°C. Statistical analysis was made using ANOVA followed by Student's t-test.

Sex	Genotype	*Average rectal temperatures at 4°C	n	P values
Males	WT	36.5 ± 1.4	7	<0.01
	UCN1KO	37.1 ± 1.0	7	
Females	WT	36.8 ± 1.1	6	<0.001
	UCN1KO	37.9 ± 0.4	6	

Table 2: UCN1KO and WT mice have similar body weights: statistical analysis was done using ANOVA followed by Student's t-test.

Sex	Genotype	Body weight (grams) means ± SEM	(n)	P values
Males	WT	24.8 ± 0.41	29	0.157
	UCN1KO	26.0 ± 0.67	33	
Females	WT	21.4 ± 0.40	24	0.214
	UCN1KO	20.7 ± 0.35	30	

mice, developed by Muglia et al. [2] and backcrossed into C57BL/6 background for more than 6 generations, were obtained from Dr. J. Majzoub, (Children's Hospital, Boston MA, USA). CRF/UCN1 double KO (dKO) mice were developed by mating male CRFKO mice with female UCN1 KO mice. Genotyping was performed by Transnetyx (Cordova, TN, USA) using mouse tissue samples analyzed by real-time PCR. The PCR primers were designed for the CRF and UCN1 loci as in Table 5.

Mice were housed in polycarbonate micro isolators with filter tops at a controlled environment of 70–74°F ambient temperature, 40–60% humidity, and 12 h/12 h light-dark cycles. Regular rodent chow, LabDiet number 5001, and water were given ad libitum. Depending on the litter size, mice were housed in either small cages which had a maximum of 4 mice per cage or large cages which had a maximum of 10 mice per cage. During experiments, mice were housed individually in small cages. The study protocol was approved by the institutional animal care and use committee (Animal Welfare Assurance number A 3310-01).

2.2. Body Weight Measurements. Mice body weights were measured using an electronic scale (Mettler PE400) with a sensitivity of 0.01 grams.

2.3. Cold Stress Experiments. Mice, 8–16 weeks old, were individually housed and exposed to 4°C or 22°C for 2 hours in the absence of food and water. Internal body temperature was measured using an electronic rectal probe (TW2-193, Braintree Scientific) every ten minutes for two hours. The mouse was restrained by the tail wrap method in which the tail is transferred from the thumb and forefinger to the fourth and fifth fingers. The tail presses into the palm of the hand and then the thumb and forefinger grasp a scuff of skin located in the occiput of the animal. The rectal probe was lubricated by olive oil and then inserted 1 cm into the mouses rectum for about 5 seconds. Temperatures were measured at 10-minute intervals during the 120-minute period. A total of 13 temperature readings were taken per mouse per experiment. Experiments were performed between 9 am and 5 pm.

Table 3: Female UCN1KO mice do not acclimatize to cold environment. Statistical comparison of average rectal temperatures, taken every 10 min during 2 h (10 to 13 measurements for each mouse, 3 to 8 animals in each group) in mice exposed 2 times to 4°C. The second time was performed 3 days after the first attempt. Statistical analysis was made using ANOVA followed by Student's t-test.

Genotype	Sex	Attempt number	Rectal temperature (°C) means ± SD	(n)	P values[(a)]
WT	Males	1st	36.9 ± 0.8	7	<0.001
		2nd	37.4 ± 0.6	7	
	Females	1st	37.3 ± 0.4	7	<0.001
		2nd	38.0 ± 0.4	3	
UCN1KO	Males	1st	37.3 ± 0.7	6	<0.05
		2nd	37.8 ± 0.6	3	
	Females	1st	37.8 ± 0.5	8	NS
		2nd	37.6 ± 0.5	6	

2.4. Dexamethasone Treatment Experiment. CRFKO and dKO mice were given daily intramuscular (IM) dexamethasone injections for three consecutive days. Each dexamethasone injection consisted of 1 μg of dexamethasone dissolved in 0.1 mL of 0.9% saline solution. On the fourth day (the day following the last injection), mice were exposed to 4°C for 2 hours, and the internal body temperature was recorded at 10 minute intervals using the same electronic rectal probe mentioned earlier.

2.5. Cold Acclimatization Experiments. To study the effects of cold environment on internal body temperature, male and female UCN1KO and WT littermate mice, 8–12 weeks old, were exposed for a second time (3 days after the first exposure) to 4°C for 2 hours; the internal body temperature was recorded at 10-minute intervals using the same method described above.

2.6. Tissue Homogenization. Skeletal muscle, subcutaneous adipose (SQ fat), visceral adipose (v fat), brown adipose (BAT), kidney, heart, and liver tissues were isolated from WT and UCN1KO male and female 8–12 weeks old C57BL/6 mice. Tissue samples, 100–200 mg, were placed in 400 μL of Trizol reagent (Invitrogen) on ice. Tissue samples were homogenized using three 5-minute cycles at max speed of a Bullet Blender with stainless steel beads (BBX24B, Next Advance, Averill Park, NY, USA) at 4°C. Trizol reagent (600 μL) was added to the homogenates. The samples were then centrifuged at 3000 g for 5 minutes at 4°C. Supernatants

Table 4: Expression levels of transcripts measured by real-time polymerase chain reaction (RT-PCR) from total RNA prepared from brown adipose tissues (BAT), skeletal muscles, kidney, subcutaneous fat (SQ fat), liver, and heart. The data are expressed as means ± SEM. *P* values of UCN1 KO versus WT were calculated using Student's *t*-test. BAT: brown adipose tissue; UCP1: uncoupling protein 1; THR-α: thyroid hormone receptor-α subunit; THR-β: thyroid hormone receptor-β subunit; AdpR1: adiponectin receptor 1; AdpR2: adiponectin receptor 2; CRFR2: corticotropin-releasing hormone receptor 2.

Gene	Tissue	Sex	Genotype	RQ	(*n*)	*P* values
UCP1	BAT	Females	WT	0.74 ± 0.38	6	0.493
			UCN1KO	0.44 ± 0.23	8	
		Males	WT	3.10 ± 1.53	9	0.802
			UCN1KO	2.61 ± 1.11	9	
THR-α	Skeletal muscle	Females	WT	2.22 ± 0.93	6	0.731
			UCN1KO	1.74 ± 0.96	7	
		Males	WT	2.40 ± 1.30	8	0.959
			UCN1KO	2.49 ± 0.94	6	
THR-β	Kidney	Females	WT	0.42 ± 0.15	6	0.864
			UCN1KO	0.39 ± 0.11	8	
		Males	WT	2.98 ± 0.86	9	0.884
			UCN1KO	3.19 ± 1.16	7	
Adiponectin	SQ fat	Females	WT	1.25 ± 0.23	6	0.547
			UCN1KO	1.48 ± 0.28	6	
		Males	WT	1.00 ± 0.30	8	0.273
			UCN1KO	1.78 ± 0.64	7	
AdpR1	Skeletal muscle	Females	WT	2.00 ± 0.29	6	0.014
			UCN1KO	1.09 ± 0.16	8	
		Males	WT	0.56 ± 0.13	9	0.905
			UCN1KO	0.60 ± 0.28	7	
AdpR2	Liver	Females	WT	0.58 ± 0.10	6	0.176
			UCN1KO	0.37 ± 0.10	8	
		Males	WT	0.67 ± 0.12	9	0.175
			UCN1KO	1.46 ± 0.54	9	
CRFR2	Heart	Females	WT	0.28 ± 0.15	6	0.368
			UCN1KO	0.96 ± 0.65	7	
		Males	WT	4.04 ± 1.21	8	0.319
			UCN1KO	6.14 ± 1.70	6	

Table 5

Gene locus	Forward primer	Reverse primer	Size of PCR product
CRF WT	GCTCAGCAAGCTCACAGCAA	GAGCTTACACATTTCGTCC	400 bp
CRF KO	ATCGCCTTCTTGACGACTTC	GAGCTTACACATTTCGTCC	600 bp
UCN1 WT	GAGGGGACGCGCTACGCTCC	GTCCGAGCTAGCTCCAGCAG	400 bp
UCN1 KO	GTCCGAGCTAGCTCCAGCAG	GCGAATGGGCTGACCGCTTC	900 bp

Table 6

Gene	Forward primer	Reverse primer
UCP1	GAAGGATTGCCGAAACTG	CAATGAACACTGCCACAC
THR-α	GTCAGACCCAGAGGAGAACAG	CACAAGTGATACAGCGGTAGTG
THR-β	CAACCAGTGCCAGGAATGTC	CGCCTCTTCTCACGGTTC
CRFR2	CTGCTTCCAGTGCTCCAGGTG	CTACTAGGGCTCCGGGTGC
Adiponectin	CACCAAAAGGGCTCAGGATGC	CTGCCATCACGGCCTGGTGTG
Adiponectin receptor 1	GCACAGTGGGACCGGTTTGC	CACCGTGGTGGCCTTGAC
Adiponectin receptor 2	GTCAGAGCAGGAGTGTTCGTG	GTGGCAGCCTTCAGGAAC
β-Sctin	GCCTTCCTTCTTGGGTATG	GATCTTGATCTTCATGGTGC

were isolated and placed in fresh 1.5 mL RNAase/DNAase-free 1.5 mL microcentrifuge tubes and stored at −80°C.

2.7. RT-PCR. Homogenized samples were thawed on ice (4°C) and RNA extraction was continued as per the manufacturer's protocol (Invitrogen). cDNA synthesis was performed using a reverse transcription system kit (Promega). Real-time PCR was performed using a 40-cycle PCR protocol using SYBR green reagent (SA Biosciences). Data were reported as relative quantity (RQ) of the amount of mRNA of interest corrected by the amount of β-actin mRNA in the same sample (see Table 4).

Specific RT-PCR primers were designed for each of the genes (see Table 6).

2.8. Statistical Analysis. The significance of change between groups, that is, between two groups, was determined using Student's t-test. Moreover, when 3 or more groups are compared, one-way analysis of variance (ANOVA) was first used. Statistical analyses were performed on SPSS. Means and standard deviations (SD) are reported in the tables, and means and standard errors of the means (SEM) are shown in the figures.

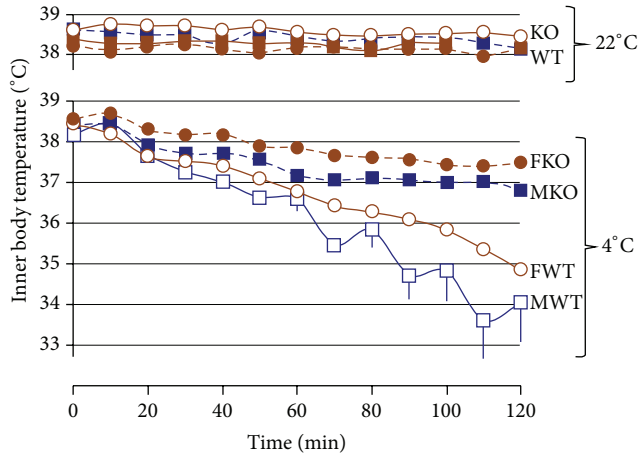

Figure 1: Rectal temperatures in wild type (WT) and UCN1 KO mice kept for 2 hours at room temperature (RT, 22°C) or at 4°C. Rectal temperature was measured with an electronic probe every 10 min. The data are means ± SEM ($n = 3$ at 22°C and $n = 6$ at 4°C) and are recorded from female WT (FWT, empty circles, solid lines), female UCN1 KO (FKO, filled circles, dotted lines), male WT (MWT, empty squares, solid lines), and male UCN1KO (MKO, filled squares, dotted lines).

3. Results and Discussion

3.1. Rectal Temperatures at 22°C and 4°C of Wild Type and UCN1KO Mice. The inner body temperature measured every 10 min in mice kept at 22°C for 2 h was stable in all the mice, regardless of genotype or sex, and was in the range of 38.1 to 38.6 (Figure 1, top panel). Male and female WT mice, kept at 4°C for 2 h, dropped their inner body temperature to 34.1 ± 2.4°C ($P < 0.001$) and 34.9 ± 3.1°C ($P < 0.001$) at 120 min, respectively, (Figure 1, lower panel). In contrast, the inner body temperature of male and female UCN1KO mice kept at 4°C for 2 h decreased only to 36.8 ± 0.7°C ($P < 0.01$) and 38.1 ± 0.5°C ($P < 0.05$), respectively (Figure 1). We also compared the averages of all values taken during the 120 min at 4°C between WT and UCN1KO mice (Table 1). The inner body temperature of UCN1KO mice was significantly higher than that of WT mice in both males and females (Table 1).

The thermoregulatory response to cold is a complex biological process that involves multiple neuroendocrine pathways [18, 19]. UCN1 is an important neuropeptide, which plays multiple roles in animal physiology particularly in the adaptive phase to stress [20]. UCN1KO mice were reported to have a sensory hearing loss with normal corticosterone level and normal adrenal response to acute restraint stress [13, 14], impaired acclimatization of corticosterone response to repeated constraints, and impaired response to cold stress [15]. Our data showing that UCN1KO mice exposed to 2 h cold environment (4°C) had a higher average core body temperature than sex- and aged-matched WT littermates suggest an important role for UCN1 in thermoregulation. Furthermore, the ability of UCN1KO mice to tolerate 4°C environment may explain our previous finding of impaired corticosterone response to cold stress in these mice. The 2 h cold exposure of the UCN1KO mice may not represent a sufficient stress to increase the HPA activity, as inner body temperature does not decline as readily as WT mice.

3.2. Body Weight of Wild Type and UCN1KO Mice. As body size may influence heat loss in the cold, we measured the body weight of the UCN1KO and WT mice. The body weights of WT mice were not different from those of UCN1KO mice: 26.0 ± 0.7 versus 24.8 ± 0.4 gr for males and 20.7 ± 0.4 versus 21.4±0.4 gr for females, respectively (Table 2). The data suggest that body weight does not contribute in the difference in core body temperatures observed in WT or UCN1KO mice kept at room temperature or at 4°C.

3.3. Rectal Temperatures at 22°C and 4°C of CRFKO and UCN1/CRF dKO Mice. To investigate the role of CRF in temperature regulation, we recorded the internal body temperature of female CRFKO and UCN1/CRF dKO mice at 22°C and 4°C for 2 h. The internal body temperatures of CRFKO and UCN1/CRF dKO mice at 22°C were stable throughout the 2-hour period (37.8 ± 0.2 and 38.1 ± 0.1°C, resp.). In contrast, at 4°C the inner body temperatures of CRFKO and UCN1/CRF dKO mice showed dramatic declines to 26.1 ± 4.9°C ($P < 0.01$) and 32.5 ± 1.8°C ($P < 0.05$), respectively (Figure 2).

Since CRF deficiency results in glucocorticoid deficiency [2], we treated CRFKO and CRF/UCN1 dKO mice with dexamethasone for 3 days and assessed their internal body temperature fluctuation at 22°C and at 4°C on day 4. Dexamethasone treatment prevented the dramatic decline in internal body temperature in both the CRFKO and the CRF/UCN1 dKO mice (Figure 2); their average body temperature was not significantly different from mice kept at 22°C.

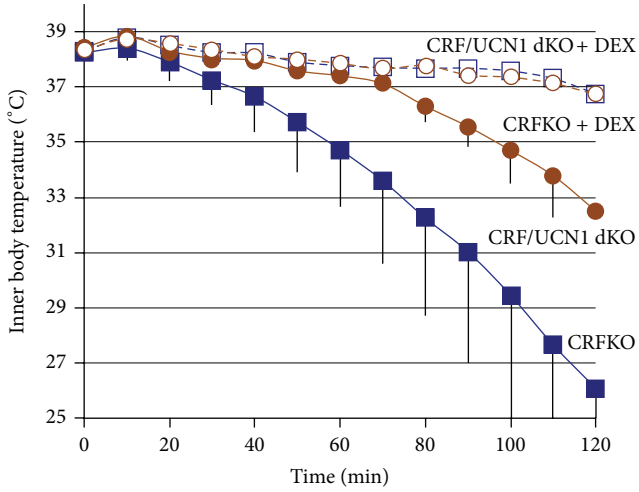

Figure 2: Responses to cold environment (4°C) in female CRFKO ($n = 4$) and CRF/UCN1 dKO ($n = 4$) mice, 12–16 weeks old, before and after treatment with 1 μg/animal/day for 3 days with dexamethasone (+DEX). Rectal temperature was measured with an electronic probe every 10 min. The data are means ± SEM.

Several reports have previously shown involvement of the UCNs/CRF peptides in thermoregulation. Intracerebroventricular injection of CRF and UCN1 in rats induced a hyperthermic response, the effects of CRF were mediated by CRFR1, whereas the effects of UCN1 were mediated by CRFR2 [21]. Intraperitoneal injection of CRF and UCN1 reduced oxygen consumption in lean and ob/ob mice [22]. Central administration of a CRF-BP ligand inhibitor in rats elevates rectal and core temperature without accompanying cardiovascular activation [23]. Mice deficient for CRFR2 showed significantly elevated basal brown fat thermogenesis and prolonged adrenergic responses in older mice and decreased respiratory exchange ratio; the latter was normalized with a CRFR1 antagonist [24].

The striking decline in core body temperature of the CRFKO mice can be attributed to either corticosterone deficiency or lack of central effects of CRF. Since the impaired tolerance to cold environment was rescued by dexamethasone treatment, it is likely that the decreased tolerance of the CRFKO mice to cold is primarily secondary to corticosterone deficiency. In this regard, glucocorticoids were reported to protect core body temperature from hypothermia induced by inhalation of a 0°C mixture of helium : oxygen (80 : 20) and to promote animal survival [25].

The CRF/UCN1 dKO mice had a lower average internal body temperature during the 2-hour cold exposure than their WT littermates but higher temperature than the CRFKO mice; this phenotype suggests different physiological roles for UCN1 and CRF in thermoregulation. Similar to our finding in the CRFKO mice, the impaired tolerance to cold stress of the CRF/UCN1 dKO mice was also reversed by a 3-day dexamethasone treatment. This further suggests that the impaired tolerance to cold stress of the dKO mice, despite being alleviated by the UCN1 deficiency, is primarily due to corticosterone deficiency.

3.4. Acclimatization of Inner Body Temperature to Repeated Exposure to Cold. Because the stress response is an adaptive phenomenon and because UCN1KO mice do not show adaptation to repeated immobilization stress [15], the acclimatization of WT and UCN1KO mice to repeated cold exposure was investigated. Exposure of male and female WT mice and male UCN1KO to 4°C for a second time (Table 3) showed that the second exposure was less effective in causing a decline in inner body temperature by about 0.5°C (Table 3). In contrast, the average inner body temperatures of female UCN1KO mice during the first exposure and the second exposure were not different (Table 3). This suggests sex difference in the UCN1KO mice acclimatization to cold environment. This contrasts with the lack of acclimatization of the corticosterone response to 15 min constraint described in both male and female UCN1KO mice [15]. Sexual dimorphism in physiological responses may be secondary to differences in sex hormones and/or in body fat composition.

3.5. Gene Expression. To probe the mechanism of the increased tolerance to cold stress in UCN1KO mice, we measured the steady-state levels of mRNA expression for selected genes thought to be involved in the regulation of thermogenesis and basal metabolic rates in white and brown adipose tissues, muscles, and liver obtained from WT and UCN1KO mice. These genes include the thyroid hormone receptors, which are known to be involved in the thermoregulatory response [26], and the uncoupling protein 1 (UCP1) which was shown to be important in the nonshivering thermoregulatory response [27]. We examined by real-time PCR the mRNA levels of UCP1 in brown adipose tissue (BAT), thyroid hormone receptor-α (THR-α) in skeletal muscles, thyroid hormone receptor-β (THR-β) in kidneys, adiponectin in subcutaneous fat (SQ-fat), adiponectin receptor 1 (AdpR1) in skeletal muscles, adiponectin receptor 2 (AdpR2) in liver, and CRFR2 in the heart. AdpR1 mRNA expression in skeletal muscle was significantly higher in female WT mice compared to UCN1KO mice (2.00 ± 0.29 versus 1.09 ± 0.16, resp.) ($P < 0.05$). No other significant difference was detected in the expression of the other genes (Table 3). AdpR1 is known to be involved in physiological functions related to insulin regulation and may have a role in thermoregulation which is yet to be discovered. Male-female differences in gene expression are not uncommon; the decreased AdpR1 expression in female UCN1KO mice may contribute to sexual dimorphism in physiological responses between male and female mice, such as lack of acclimatization to the cold of the female mice (Table 2).

4. Conclusion

Both UCN1 and CRF play important roles in the thermoregulatory response to cold. The role of CRF seems to be secondary to adrenal corticosterone. The role of CRF and glucocorticoid in thermoregulation is not surprising as they have dramatic effects on the metabolism of carbohydrate, fat, and protein; however, our findings suggest a novel role for UCN1 in thermoregulation that has not been previously recognized.

UCN1 may modulate the central thermoregulatory circuits or may influence basal metabolic rate and/or heat generation, as CRFR1 and CRFR2 are widely distributed in the CNS and in the periphery.

Authors' Contributions

Bayan Chaker and Tareq A. Samra contributed equally.

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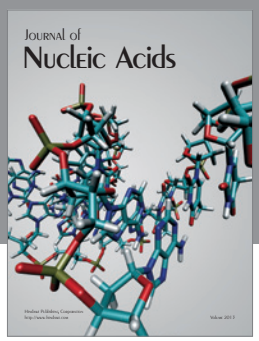

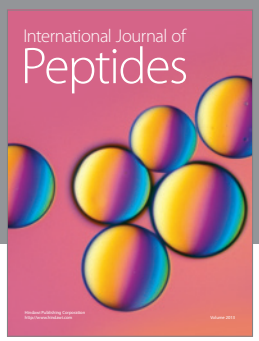

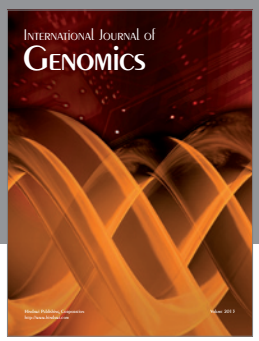

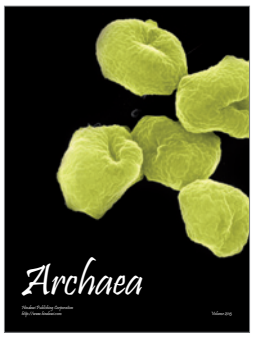

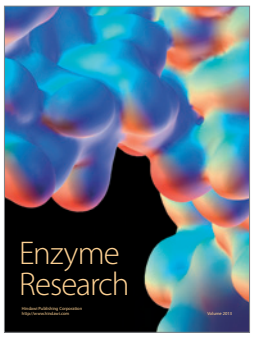

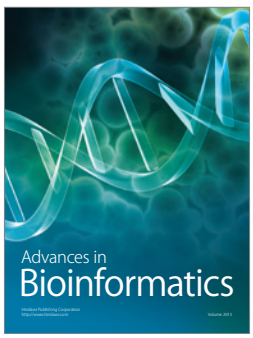

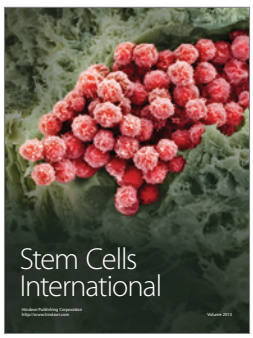

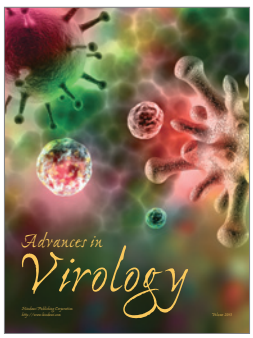

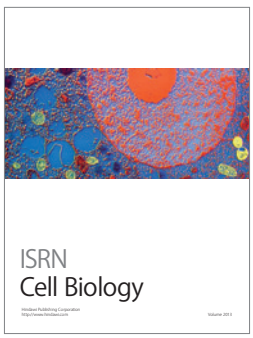

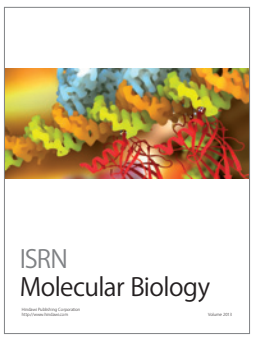

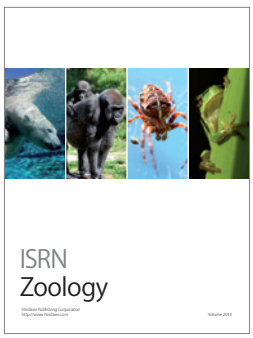

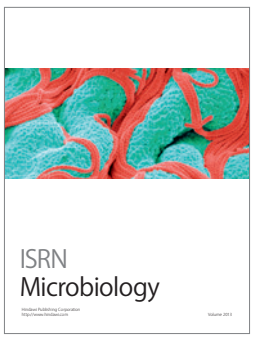

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