Re-evaluation of apparent hypometabolism in iron-deficient rats

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Experiments were performed to characterize body temperature, metabolic rate, plasma thyroid hormone concentrations, and nonshivering thermogenesis in iron-deficiency anemia at several environmental temperatures (32°, 25°, 20°, 15°, 10°, 8° C). Iron-deficient anemic rats (hemoglobin <60 g/L) were significantly colder than controls after 48-hr exposure to the lowest three temperatures despite having similar metabolic rates per $kg^{0.75}$ body weight (BW). These anemic rats were also able to increase metabolic rate 28% above control rats in response to a pharmacologic dose (75μg/100 gm BW) of norepinephrine. Brown adipose tissue mitochondrial ^{3}H-GDP binding was significantly higher in iron-deficient animals than control animals at 25° C, but was significantly lower in iron-deficient animals at lower temperatures due to smaller fat pads and slightly lower mitochondrial protein contents. Thyroid hormone status varied with severity of iron deficiency such that less severely anemic iron-deficient rats with improved thyroid status had an improved thermoregulatory performance. The insufficient metabolic response to cold in iron deficiency is seemingly related to both a thyroid hormone limitation for nonshivering thermogenesis as well as a sympathetic nervous system defect because the pharmacologic dose of norepinephrine was able to increase metabolic rate.

Keywords: GDP binding; anemia; thermoregulation; rats; cold stress; metabolic rate; norepinephrine

Introduction

Investigations over the last 10 years have shown that iron-deficient anemic rats fail to thermoregulate when exposed to air temperatures below 10° C.[1–4] The severity of the thermoregulatory defect was directly correlated with the severity of anemia and was attributed to a poor thyroid hormone response.[1] This altered thyroid state was further characterized by decreased plasma triidothyronine (T_3) disappearance,[5] low plasma hormone concentrations,[1,2,6] and a decreased hepatic production of T_3 from thyroxine.[6] Some of these alterations are similar to those seen in thyroid metabolism during prolonged fasting, protein-energy malnutrition, and manipulation of the composition of the diet.[7] That is, there is a decrease in plasma total and free thyroid hormones as well as utilization rates of T_3 during caloric deprivation.[8,9] Fasting, however, is associated with a decreased sympathetic nervous system activity while hypothyroidism is associated with a compensatory increase in sympathetic activity.[10] Hypothyroidism limits the capacity for nonshivering thermogenesis in rats at low temperatures by limiting the production of intracellular brown fat T_3, nuclear occupancy of T_3 receptors, and the required increase in mRNA for uncoupling protein (UCP).[11–13]

Dillman[3] noted that iron-deficient anemic animals had higher metabolic rates than control animals at environmental temperatures of 24°–26° C and lesser metabolic rates at 4° C. The latter observation is consistent with demonstrations of hypothyroidism and hypometabolism in iron-deficient rats[5,6] and humans,[14] while the former observation is not. Other experiments revealed that norepinephrine (NE) turnover in interscapular brown adipose tissue was not increased in anemic animals at 10° C for 7 days relative to turnover at 24° C,[2] demonstrating a blunted activity of the sympathetic nervous system to activate nonshivering thermogenesis.[15] Metabolic rate was not measured in those cold-stress studies nor have other indices of nonshivering thermogenesis, such as ^{3}H-GDP binding to brown fat mitochondria, been measured in iron-deficient animals acutely exposed to cold. The binding of ^{3}H-GDP

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to brown fat mitochondria is proportional to the activation of nonshivering thermogenesis in rat brown fat and can thus be used to estimate the potential of that tissue for heat production.[11]

With these thoughts in mind, we performed several experiments to determine if poor thermoregulation in iron-deficiency anemia could be due to an impaired capacity to increase metabolic rate and nonshivering thermogenesis due to the poor thyroid status in iron-deficiency anemia. Because the severity of iron deficiency and the environmental temperature are both key factors determining the extent of the thermoregulatory failure, we use both of these factors in our research designs.

Materials and methods

Dietary protocol

Male rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) at 21 days of age and were randomly divided equally into iron-deficient and iron-sufficient dietary treatment groups. They were housed in stainless-steel wire mesh cages in a temperature controlled facility (25° ± 1° C) and exposed to a 12-hr light:dark cycle beginning at 08:00. Both groups were fed a purified diet, meeting the recommendation of the American Institute of Nutrition (AIN 76A), except for the iron content in the low-iron diet and the omission of cellulose due to variable iron contamination. The low iron diet contained approximately 2 ppm iron. The control diet was identical to the iron-deficient diet except for the addition of 50 ppm iron added as ferrous sulfate. Animals were fed their appropriate diets and distilled deionized water ad libitum.

Experiment Ia

After 6 weeks on dietary treatment, animals were sequentially exposed to 32°, 20°, 15°, 10°, and 8 ± 1° C for 48 hr at each temperature. Animals were placed in the environmental chamber at 08:00 for a 24-hr period prior to the metabolic rate determinations that began at 08:00 the next day. Food was available at all times except for 8–9 hours before and during metabolic rate measures. Rectal temperatures were monitored at 15° C and below as an index of core temperature. Rectal temperatures were measured just prior to metabolic rate measurements using a model 43TA Tele-thermometer (Yellow Springs Instrument Co., Yellow Springs, OH, USA). During the week prior to various temperature exposures, minimum metabolic rate was measured as estimated by metabolic rate during pentobarbital anesthesia *(Table 1)*. This measurement is thought to reflect predominately thyroid-based metabolism.[16]

Experiment Ib

Based on observations of hypothermia in anemic animals exposed to temperatures below 15° C, we wanted to examine ^{3}H-GDP binding to brown fat mitochondria in anemic and control animals exposed to 10° C for 48 hr or living at 25° C. The cold-stress protocol was exactly as defined in experiment Ia, except we killed the rats by decapitation at 08:00 after 48 hr at 10° C. Rectal temperature was obtained at 08:00 each morning and before a 2-hr metabolic rate measurement done at 08:00 after 24 hr of cold exposure. Blood was also analyzed for plasma thyroxine and T_3 concentrations. Animals used at room temperature were sacrificed in the early morning and in an 8–9 hr fasted state.

Table 1 Effects of anesthesia and norepinephrine on metabolic rate

	Metabolic rate	
	Iron-deficient	Control
Minimum[a] (mL O_2/min/kg$^{0.75}$)	12.1 ± 2.1	12.0 ± 1.9
kcal·hr^{-1}·kg$^{-0.75}$	3.57 ± 0.61	3.59 ± 0.51
Maximum[b] (mL O_2/min/kg$^{0.75}$)	46.5 ± 5.3*	36.3 ± 4.6
kcal·hr^{-1}·kg$^{-0.75}$	13.57 ± 1.55	10.59 ± 1.27

[a]Measured while animals were anesthetized with pentobarbital and body temperature maintained at 37.5° C.
[b]Measured after animals were injected with 75 μg norepinephrine/100 g BW.
*Significantly different than controls, $P < 0.05$.

Experiment II

To examine the impact of a slightly less severe degree of iron-deficiency anemia on thermoregulatory performance, metabolic rate, and the impaired ^{3}H-GDP binding we repeated experiment Ib at five temperatures with animals receiving only 24 days of dietary treatment. Animals were exposed to one of five different temperatures (32°, 20°, 15°, 10°, 8° C) for 48 hr beginning at 08:00. As before, all rats were allowed a 24-hr acclimation period at each temperature and provided free access to food and water up to 8–9 hours before the metabolic rate was measured beginning at 08:00. As in experiment I, we then measured resting metabolic rates during the second 24-hr period at each temperature. Rectal temperatures were measured at 08:00 each morning of the cold exposure in the 9-hr fasted state. This coincided with the time the animals were placed in the calorimeter for metabolic rate measurements on day 2 and the time of sacrifice for GDP-binding studies on day 3.

Biochemical methods

Animals were sacrificed by decapitation at 08:00 of day 3 at each temperature. Organs were quickly removed and brown adipose tissue prepared for measurement of ^{3}H-GDP binding by described methods.[17] Plasma T_3 and thyroxine (T_4) from the trunk blood were measured by radioimmunoassay (Monobind Corp., Costa Mesa, CA, USA), hemoglobin by standard cyanmethemoglobin, and hematocrit by microcapillary methods.

Expired gas determinations

Metabolic rate and carbon dioxide production were measured in four animals simultaneously for a 2-hr period using a computer-controlled rapid flow indirect calorimeter.[18] Animals were allowed to acclimate for 30–60 minutes prior to actual data collection. These measurements were begun at 08:00 in the light cycle. Air was pulled through the chambers at 1.9 liters per minute, dried on Drierite (W.A. Hammond Drierite Co., Xenia, OH, USA), and a portion was then diverted in parallel (125 mL/min) into an Applied Electrochemistry S-3A oxygen analyzer (Sunnyvale, CA, USA) and an Ametek CD-3A carbon dioxide analyzer (Thermox, Pittsburgh, PA, USA). Data were logged via digital-analog conversion onto a personal computer. Each metabolic chamber was sampled for 3 minutes followed by room air sampling

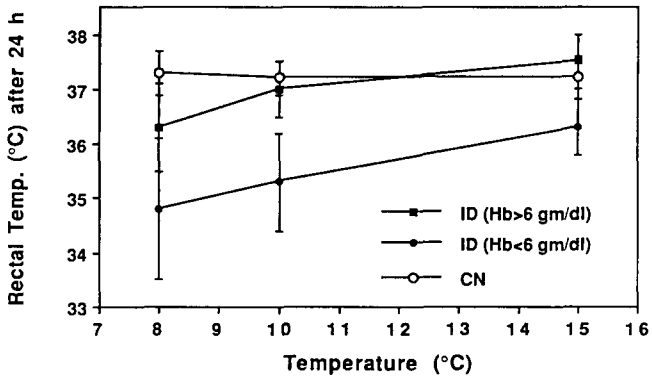

Figure 1 The rectal temperatures of iron-deficient and control rats (n = 10/group) following 24-hr exposure to 15°, 10°, and 8° C. The iron-deficient rats have significantly lower body core temperatures at all three environmental temperatures compared with control rats ($P < 0.05$) if the hemoglobin is <6 g/L and at 8° C if Hb > 6 g/L.

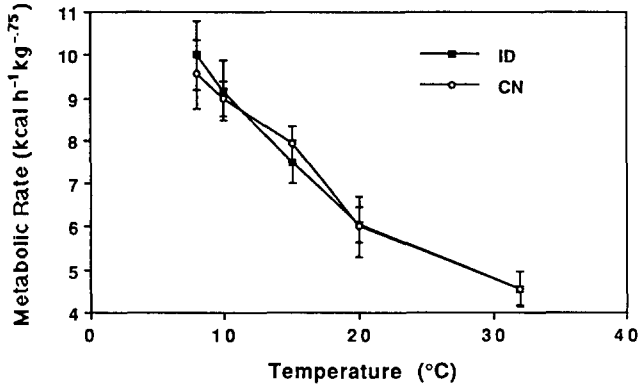

Figure 2 The metabolic rates (kcal/kg$^{0.75/hr}$) of two groups of rats fed either iron adequate (CN) or iron deficient (ID) diets. The values represent the average metabolic rate for each group (n = 10/group) exposed sequentially to 32°, 20°, 15°, 10°, and 8° C for 48 hr. The values were obtained following 24-hr exposure to each temperature. Metabolic rates for iron deficient rats are not significantly different from control rats ($P < 0.05$).

(also for 3 minutes) to allow for analyzer equilibration. A total of eight three-minute samplings were made for each chamber and room air with individual readings being taken every 15 seconds. Metabolic rate was calculated using standard equations.[19] These data were non-normally distributed within animals and the average of the lowest quartile of the individual rat's metabolic rate measurements were taken to estimate resting metabolic rate.[20] All animals were fasted approximately 9 hr prior to metabolic rate determinations and had access to food for at least 3 hr in the beginning of the previous dark cycle.

Statistical analysis

Data were analyzed by a two-way analysis of variance (ANOVA) with temperature and iron status being the main effects. Possible interaction terms were considered and specific post-hoc analysis conducted by least squares-means comparisons on the general linear models routine on SAS (Statistical Analysis System, Cary, NC, USA).

Results

Experiment Ia

Following 6 weeks of dietary treatment, iron-deficient animals (n = 10) were significantly smaller than control animals and were severely anemic. They had an average hematocrit value of 16% ± 2% and a hemoglobin concentration of 41 ± 7 g/L, while control animals (n = 10) averaged 45% packed cell volume (PCV) and 144 ± 4 g/L. The average body weight of the iron-deficient group (151 ± 17 gms) was significantly lower than the control group (208 ± 14 g, $P < 0.05$) after 6 weeks of dietary treatment.*

Control animals maintained core body temperature of approximately 37° C, while iron-deficient anemics showed significant losses of core body temperature upon exposure to 15° C, 10° C and 8° C *(Figure 1)*. Forty percent of iron-deficient anemic animals were removed from the 8° C exposure because core temperature fell below 30° C. Metabolic rate was unaffected by iron deficiency when expressed relative to metabolic body size (kg$^{0.75}$) *(Figure 2)*. The mean of the bottom quartile of each animal's metabolic rate measurement, an estimate of "resting" metabolic rate was also unaffected by iron deficiency.

There was no difference in minimum metabolic rate/kg$^{0.75}$ in iron-deficient versus control animals under pentobarbital anesthesia *(Table 1)*. However, when animals were injected with 75 μg norepinephrine/100 g BW intraperitoneal to elicit a maximal metabolic response, metabolic rate/kg$^{0.75}$ was significantly (28%) higher in iron-deficient animals than controls *(Table 1)*.†

Experiment Ib

Iron-deficient animals exposed to 10° C for 48 hr had a decline in core temperature of 2.0 ± 0.4° C, compared to 0.2 ± 0.1° C in controls. They also had significantly lower ^{3}H-GDP binding than controls (1221 pmol/pad versus 2298 pmol/brown fat pad) though specific ^{3}H-GDP binding was not much different *(Table 2)*. Plasma T_3 and T_4 were also significantly lower in iron-deficient rats than controls. In contrast, iron deficient rats at 25° C had significantly higher GDP binding than controls *(Table 2)*.

Experiment II

Since hypothermia was so pronounced in the anemic animals in the first study, we choose to examine aspects

*With exposure to temperatures of 15° C and below, the iron-deficient animals began losing weight with an average loss of 7 g at 15° C, 6 g at 10° C, and 7 g at 8° C for each 48-hr period. In contrast, control animals gained 3–5 g.

†Dose-response curves for both iron-deficient and control animals were determined in preliminary tests. Maximal metabolic rates were obtained at 35–50 μg NE/100 g in both groups.

Table 2 Control and iron-deficient rats at room temperature or 10° C

	Iron-deficient	Control
	(n = 9)	(n = 11)
25° C		
Body weight (g)	254 ± 32	358 ± 26*
Hematocrit (% PCV)	16 ± 2	48 ± 3*
IBAT		
Weight (mg)	640 ± 130	700 ± 80
Mitochondrial protein[b] (mg/g BAT)	5.64 ± 1.01	5.78 ± 0.88
^{3}H-GDP binding (pmoles/mg protein)	123 ± 29	89 ± 22*
	(n = 8)	(n = 8)
10° C for 48 hrs		
Body weight (g)	180 ± 16	258 ± 8*
Hemoglobin (g/L)	49 ± 6	142 ± 4*
Plasma T_3 (ng/mL)	49 ± 13	96 ± 15*
Plasma T_4 (μg/dL)	1.7 ± .4	2.6 ± 0.3*
IBAT		
Weight (mg)	520 ± 67	807 ± 113*
Mitochondrial protein (mg/g BAT)[a]	7.41 ± .79	8.00 ± 0.58
^{3}H-GDP binding (pmoles/mg protein)	317 ± 35	356 ± 51

Note: Table of means ± SD.
*Significant difference between dietary treatments, $P < 0.05$.
[a]Mitochondrial protein was determined as the yield of protein from centrifugal purification.

of nonshivering thermogenesis in animals less severely affected. In this study hemoglobin concentration averaged 65 ± 6 g/L in iron-deficient animals and 130 ± 10 g/L in controls after 24 days of dietary treatment. Thus, a shorter period of dietary treatment provided a less severe decline in hemoglobin. There was a significant difference in body weight between iron-deficient and control groups (167 ± 5 g versus 205 ± 7 g, $P < 0.01$.

These less anemic rats were able to maintain a higher core temperature at 8° C and 10° C than the severely anemic animals in the initial study. Core temperature fell to 36.3 ± 0.4° C at 8° C and to 37 ± 0.5° C at 10° C compared to 38.0 ± 0.3° C in controls at each of these temperatures. As before, metabolic rate was similar in anemics and controls at each temperature and to the animals studied in experiment I.

We again observed that ^{3}H-GDP binding per mg of brown adipose tissue (BAT) mitochondria protein was similar in iron-deficient and control rats *(Table 3)*. However, a consistently smaller brown fat pad per g of body weight and a slightly smaller mitochondrial protein content per pad contributed to an average 23% lower GDP binding per pad.

There was little effect of these acute changes in temperature on plasma thyroxine concentration with control animals averaging 2.8 ± 0.2 μg/dL and iron-deficient animals averaging 2.2 ± 0.2 μg/dL ($P < 0.05$). There was little effect of temperature on plasma T_3 except for an expected increase ($P < 0.001$) from 44 ± 10 ng/dL to 71 ± 10 ng/dL as the temperature decreased from 32° C to 20° C. Thereafter, control rats averaged 74 ± 8 ng/dL and iron-deficient animals only 58 ± 8 ng/dL ($P < 0.05$).

Discussion

These experiments demonstrate that iron-deficiency anemia in young rats is associated with hypothermia at low environmental temperatures despite metabolic rates that would appear normal based on the animal's body surface area. Because hypothermia occurred, heat production or heat distribution mechanisms were inappropriate for the heat loss rates of iron-deficient anemic animals. The conclusion of limited heat production is supported by the observation of significantly less GDP binding per fat pad. This expression is what Cannon and Needergard regard as the best index of thermogenic capacity of nonshivering thermogenesis.[11] Animals who were not as severely anemic due to a lesser period of dietary iron restriction had a smaller thermoregulatory defect, a lesser deficit in GDP binding per fat pad, and a better thyroid status. Thus, the severity of the anemia and duration of iron deprivation to peripheral organs[21] are critical to the balance between heat production and loss. There was no effect of iron deficiency on the minimal metabolic rate. That is, the metabolic rate when nervous system control of metabolism is removed and the predominate influences are from thyroid hormone. In contrast, maximal metabolic rate was elevated in iron-deficient animals and demonstrates an enhanced sensitivity to norepinephrine. This enhanced sensitivity to norepinephrine may be a result of both thyroid status, and regulation of adrenergic receptors.[15,16] Because an injection of a large dose of norepinephrine dramatically increased metabolic rate in the iron-deficient anemic rats and the brown adipocyte is a primary target for this neurotransmitter; we infer that a defect in post-adrenoceptor

Table 3 Characteristics of interscapular brown adipose tissue

Iron group		Iron-deficient (n = 8-9)	Control (n = 8-9)
32° C			
	BAT wt (mg)	0.62 ± 0.18	0.67 ± 0.14
	Mitochondrial protein (mg)	2.27 ± 0.54	2.50 ± 0.42
	GDP bound: pmoles/mg protein	97 ± 27	128 ± 40
	pmol/IBAT pad	219 ± 37	284 ± 38*
20° C			
	BAT wt (mg)	0.50 ± 0.10	0.65 ± 0.10
	Mitochondrial protein (mg)	3.32 ± 0.41	4.28 ± 0.67
	GDP bound: pmoles/mg protein	314 ± 44	304 ± 60
	pmol/IBAT pad	970 ± 110	1300 ± 314
15° C			
	BAT wt (mg)	0.54 ± 0.05	0.72 ± 0.05*
	Mitochondrial protein (mg)	3.62 ± 0.27	4.54 ± 0.53
	GDP bound: pmoles/mg protein	317 ± 55	293 ± 47
	pmol/IBAT pad	1135 ± 191	1335 ± 156*
10° C			
	BAT wt (mg)	0.49 ± 0.06	0.61 ± 0.10
	Mitochondrial protein (mg)	3.63 ± 0.37	4.88 ± 0.38
	GDP bound: pmoles/mg protein	312 ± 49	319 ± 34
	pmol/IBAT pad	1115 ± 150	1571 ± 172*
8° C			
	BAT wt (mg)	0.47 ± 0.06	0.62 ± 0.13
	Mitochondrial protein (mg)	2.92 ± 0.41	4.14 ± 0.42
	GDP bound: pmoles/mg protein	404 ± 59	362 ± 58
	pmol/IBAT pad	1184 ± 248	1529 ± 203*

Values are expressed as means ± SD. Mitochondrial protein is the yield per pad. GDP binding is expressed both as specific binding (e.g., pmoles GDP bound/mg mitochondrial protein) and as total binding per brown fat pad. Significant differences between iron-deficient and control rats are denoted by an asterisk (*) ($P < 0.05$ by ANOVA).

events in the brown adipocyte is not the only factor limiting nonshivering thermogenesis in the rats. While GDP binding per unit of mitochondrial protein was only affected by iron deficiency in its severest form, GDP binding per brown fat pad was decreased 44% in severely anemic rats and 23% in less severely deficient animals. This decrease in activation of heat production may be related to blood flow, adrenergic input nerve traffic, substrate utilization, and the amount of UCP within the mitochondria.[11]

Hypothyroidism is known to limit the capacity of the sympathetic nervous system to activate UCP synthesis in brown fat.[13,15] Hypothyroid rats are unable to increase mRNA for UCP in response to cold stress in the absence of adrenergic stimulation.[22,23] Others have failed to observe any decrease in brown fat UCP in hypothyroid animals. Perhaps the insufficient heat production in iron-deficient animals in this study is related to a poor thyroid hormone state and its influence on UCP synthesis. An increased nonshivering thermogenesis in iron-deficient animals at 25° C is consistent with a hypothyroid state and an increased turnover of brown fat norepinephrine.[11,15] This turnover of norepinephrine is thought to represent the rate of utilization of the neurotransmitter in a tissue, and thus gives a better estimate of adrenergic activity in brown fat than measurements of plasma catecholamines.[15] When iron deficient animals are exposed to colder temperatures of 10° C for 7 days, they have a blunted sympathetic nervous system drive to brown fat as measured by norepinephrine turnover.[2] While this decreased sympathetic nervous system activity at 7 days does not necessarily mean it is decreased at 2 days, the results are consistent and suggest a possible explanation for the fact the iron-deficient animals thermoregulate poorly despite the capacity to increase total body heat production to the control level when exogenous neurotransmitter is used. A low adrenergic input traffic to the brown adipocyte may also limit thyroid action within these cells because the activity of the brown fat Type II-5′ deiodinase, is significantly blunted in iron-deficient rats after 24 hr of cold stress.[24] The amount of this enzyme is strongly controlled by α_1 receptor activation by norepinephrine.[13] A limited capacity to maintain a sufficient saturation of brown fat nuclear receptors due to poor T_3 production rates within the brown adipocyte is thus a reasonable but untested explanation.

This study demonstrates that the thermoregulatory defect in iron-deficiency anemia is in part due to a limited thermogenic capacity in brown fat. Moreover, this thermogenic defect is responsive to severity of anemia. The other component of the thermal balance equation is heat loss. While measurements of heat loss in iron-deficient animals are limited, direct calorimetry experiments do show greater rates of heat loss.[25] The amount of fur and its thermal qualities have only been qualitatively evaluated.[4] Blood flow studies show higher rates of blood flow to the skin surface in anemic animals with cold exposure than in controls (unpublished

data) and we assume this would translate into greater rates of heat transfer to the environment. What remains unclear is why anemic animals cannot increase heat production beyond the amounts measured to maintain thermal balance.

In conclusion, severe iron-deficiency anemia is associated with a significant hypothermia at environmental temperatures below 15° C. This hypothermia is seemingly caused by an insufficient increase in metabolic rate or nonshivering thermogenesis to balance heat losses. Poor thyroid function in conjunction with altered central control may be responsible.

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