The activation of the arginine–citrulline cycle in macrophages from the spontaneously diabetic BB rat

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The activity of the arginine–citrulline cycle was investigated in macrophages from the spontaneous immunologically mediated diabetic BB rat. Peritoneal macrophages were prepared from male diabetes-prone (BBdp), diabetic (BBd) and age-matched non-diabetes-prone (BBn) rats. Cells were incubated at 37 °C for 2 h in Krebs–Henseleit bicarbonate buffer containing 0.5 mM L-arginine, 0.1 mM L-[*ureido*-^{14}C]citrulline and 5 mM D-glucose to measure the activity of the arginine–citrulline cycle. The uptakes of citrulline and arginine by macrophages were measured during a 5 min incubation period with L-[*ureido*-^{14}C]citrulline and L-[2,3-^{3}H]arginine respectively. The production of NO_3^- (the major stable oxidation product of NO) increased ($P < 0.01$) by 112% and 151% in 75-day-old BBdp and 115-day-old BBd macrophages respectively, compared with age-matched BBn cells. The conversion of [^{14}C]citrulline into [^{14}C]arginine increased ($P < 0.01$) by 704%, 892% and 904% in 50- and 75-day-old BBdp and 115-day-old BBd macrophages respectively, compared with age-matched BBn cells. The enhanced NO synthesis in BBdp and BBd macrophages was associated with a 25–35% increase in the uptake of L-arginine. However, there were no differences in the uptake of citrulline between BBdp or BBd macrophages and age-matched BBn cells. Our results demonstrate for the first time the activation of the arginine–citrulline cycle in macrophages in an autoimmune condition. The inherent increase in the recycling of L-citrulline to L-arginine in BBdp and BBd macrophages may reflect an innate metabolic disorder in these cells. This increased L-arginine synthesis from L-citrulline may play a role in sustaining a sufficient intracellular L-arginine concentration for prolonged generation of NO in BBdp and BBd macrophages. A role for NO in the autoimmune destruction of pancreatic β-cells in insulin-dependent diabetes mellitus warrants further investigation.

INTRODUCTION

NO, a small free-radical molecule with enormous versatility and importance, has recently united traditionally diversified research areas in biochemistry, physiology, immunology and neuroscience [1,2]. NO is derived from L-arginine by oxidation of one of the two chemically equivalent guanido nitrogens in the presence of NADPH, with L-citrulline as a co-product of NO synthase [3]. The NO synthase present in macrophages is cytokine-inducible and Ca^{2+}/calmodulin-independent, in contrast with the constitutive NO synthase in endothelial cells. Beyond its key roles as the endothelium-derived relaxing factor [4] and as a neurotransmitter [5], NO helps to kill foreign pathogenic organisms in immune responses [6]. However, excessive production of NO can be extremely destructive, particularly in an autoimmune condition [7]. On the basis of the findings that activated macrophages kill normal rat pancreatic β-cells *in vitro* via arginine-dependent NO generation [8] and that specific inhibitors of NO synthase prevent streptozotocin-induced diabetes in the mouse *in vivo* [9], NO has recently been implicated to mediate the destruction of β-cells in insulin-dependent diabetes mellitus [7,10]. However, whether macrophages increase NO production in an autoimmune condition is not known.

The spontaneously diabetic BB rat is an excellent animal model of the human insulin-dependent diabetes mellitus [11]. The disease is believed to result from the selective autoimmune destruction of β-cells by cell-mediated and/or humoral immune responses [12]. Both sexes are affected, with the incidence of diabetes beginning around the age of sexual maturation and reaching a peak at 80–100 days. The diabetic syndrome is characterized by many features of autoimmunity, including intense infiltration of islets by mononuclear cells (insulitis) including macrophages, the presence of circulating autoantibodies against islet cells, and adoptive transfer of insulitis and diabetes with activated BB rat mononuclear cells [11,12]. The essential role for macrophages in the initiation of the autoimmune destruction of β-cells in the BB rat has been strongly implicated by the following lines of evidence. First, most of the infiltrating mononuclear cells at the early stage of insulitis in the BB rat are macrophages [13]. Second, intraperitoneal administration of silica, which is selectively toxic to macrophages and depletes their numbers, to diabetes-prone BB rats prevents insulitis and diabetes [14].

There exists an arginine–citrulline cycle (the synthesis of NO plus L-citrulline from L-arginine and the conversion of L-citrulline into L-arginine) in macrophages, whose activity is markedly enhanced in activated cells [15]. The synthesis of L-arginine from L-citrulline in macrophages has been suggested to play an important role in maintaining intracellular L-arginine availability for NO generation under such a condition as inflammation, where extracellular L-arginine concentration is exceedingly low due to the activity of arginase [15]. In view of the metabolic and immunological activation of BBdp and BBd rat macrophages [16–18], we postulated that the arginine–citrulline cycle may be activated in macrophages from the spontaneously diabetic BB rat. This hypothesis was tested in the present study, by using peritoneal macrophages.

MATERIALS AND METHODS

Chemicals

L-[*ureido*-^{14}C]Citrulline, L-[2,3-^{3}H]arginine, D-[1-^{14}C]mannitol and D-[2-^{3}H]mannitol were obtained from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.). L-Citrulline,

Abbreviation used: KHB buffer, Krebs–Henseleit bicarbonate buffer.

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L-arginine, D-glucose, arginase (from bovine liver), urease (from jack beans; type VI), BSA (essentially fatty-acid-free), sulphanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, Hepes and Hyamine hydroxide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nitrate reductase and NADPH were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Ficoll–Hypaque (*d* 1.077) was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Dowex AG 50W-X8 (200–400 mesh, Na^+ form) was purchased from Bio-Rad (Richmond, CA, U.S.A.).

Animals

Male BBd rats (85–100 days old), BBdp rats (35–40 days old) and age-matched BBn rats were obtained from the Animal Resources Division, Health Protection Branch, Ottawa, Ont., Canada. Rats were housed in a light (12 h-light/12 h-dark cycle)- and climate-controlled facility in stainless-steel cages. Animals were provided *ad libitum* with laboratory rat chow (Hardland-Teklad, Bartonville, IL, U.S.A.) and water. Diabetic rats were maintained on single daily subcutaneous injections of 2–4 units of ultralente insulin (Eli Lilly, Indianapolis, IN, U.S.A.) to prevent ketosis and hyperglycaemia. Treatment of diabetic BB rats with insulin is required for their survival [11]. BBd (115 days old, 7–15 days post onset), BBdp (50 and 75 days old) and age-matched BBn rats were used for preparation of macrophages. Blood was drawn from the tail vein of the box-restrained unanaesthetized rats by using a microhaematocrit. Serum glucose was determined by an enzymic method involving hexokinase and glucose-6-phosphate dehydrogenase [19]. Serum glucose concentrations (mM; mean ± S.E.M., $n = 15$) at the time of preparing macrophages were 5.16 ± 0.29, 5.24 ± 0.32 and 4.98 ± 0.21 respectively for BBn, BBdp and insulin-treated BBd rats. Urine glucose and ketone tests were negative in 50-day-old BBdp, 75-day-old BBdp and insulin-treated BBd rats at the time of preparing macrophages, as determined by Chemstrip for urinalysis (Boehringer Mannheim). The experiments were carried out in accordance with the guidelines of the U.S. National Research Council for the care and use of laboratory animals.

Preparation of peritoneal macrophages

Rats were anaesthetized with CO_2 and killed by cervical dislocation. Peritoneal macrophages were obtained from the animals and purified on a Ficoll–Hypaque gradient (*d* 1.077) as previously described [16]. Cells were suspended in oxygenated (O_2/CO_2, 19:1) Krebs–Henseleit bicarbonate (KHB) buffer (pH 7.4) containing 5 mM glucose, 20 mM Hepes and 1% BSA [15]. Cell viability, as assessed by 0.2% Trypan Blue exclusion, was greater than 96%, and results are expressed per 10^6 viable cells.

Determination of NO synthesis and the conversion of citrulline into arginine in macrophages

Macrophages (3×10^6 cells/ml) were incubated at 37 °C for 2 h in 25 ml silicone-treated Erlenmeyer flasks containing 2 ml of oxygenated (O_2/CO_2, 19:1) KHB buffer plus 0.5 mM L-arginine, 0.1 mM L-[*ureido*-^{14}C]citrulline (1 μCi/μmol), 5 mM D-glucose, 20 mM Hepes and 1% BSA [15]. Incubations were initiated by addition of cells. Flasks were gassed with O_2/CO_2 (19:1) for 20 s before sealing with rubber stoppers. The incubations were terminated by addition of 200 μl of 1.5 M $HClO_4$. Parallel incubations, in which media were acidified with 200 μl of 1.5 M $HClO_4$ before addition of cells, were run as blanks. The acidified cell extracts were neutralized with 100 μl of 2 M K_2CO_3, and the supernatants were used for the analysis of [^{14}C]arginine and NO_3^- (nitrate). [^{14}C]Arginine was measured as $^{14}CO_2$ by a coupled enzyme assay involving arginase and urease, whereas NO_3^- was converted into NO_2^- by nitrate reductase, followed by reaction of NO_2^- with the Griess reagent [15]. It was demonstrated in preliminary experiments that the rate of L-arginine synthesis from L-citrulline was fairly constant during a 2 h incubation period.

We found that the amount of $^{14}CO_2$ produced presumably from [^{14}C]urea in the presence of added urease, but in the absence of added arginase, was about 2–3% of that in the presence of added arginase and urease. This suggests that the [^{14}C]arginine produced from [^{14}C]citrulline was substantially diluted by the large amounts of added unlabelled L-arginine (1000 nmol) present in the incubation medium, thus minimizing the conversion of [^{14}C]arginine into [^{14}C]urea plus ornithine.

Measurement of intracellular specific radioactivity of [^{14}C]citrulline in macrophages

Macrophages (5×10^6/ml) were incubated for 0.5, 1 and 2 h in the presence of 0.1 mM L-[*ureido*-^{14}C]citrulline (1 μCi/μmol) plus 0.5 mM L-arginine as described above. At the end of the predetermined incubation period, the cell suspension was transferred to a silicone-treated tube and centrifuged for 1 min at 200 *g*. The cell pellet was washed twice with 1 ml of KHB buffer containing no amino acids, by centrifugation for 1 min at 10000 *g* in a microcentrifuge. The cell pellet was suspended in 0.4 ml of KHB buffer. This cell suspension was immediately transferred to a 1.6 ml microcentrifuge tube, which contained 0.7 ml of a mixture of oil (bromododecane/dodecane, 20:1, v/v) overlaid on 0.2 ml of 1.5 M $HClO_4$. Cells were separated from the medium through the oil layer into the acid layer by centrifugation (10000 *g*, 1 min). After the oil layer had been washed three times with fresh KHB buffer, the oil layer was removed and the acid layer was neutralized with 100 μl of 2 M K_2CO_3. Preliminary experiments using D-[^{3}H]mannitol as an extracellular marker established that these washing and centrifugation procedures effectively separated cells from incubation media. The neutralized cell extract was applied to a Dowex AG 50W-X8 (200–400 mesh, Na^+ form) resin column (0.55 cm × 8.0 cm), which was eluted with water. The first 2 ml fraction containing [^{14}C]citrulline but no [^{14}C]arginine was collected. A portion of the citrulline fraction was used for measurements of [^{14}C]citrulline radioactivity and of citrulline by h.p.l.c. [20]. The specific radioactivity of [^{14}C]citrulline was calculated by dividing the amount of [^{14}C]citrulline radioactivity (d.p.m.) by the amount of citrulline (nmol).

We were aware that [^{14}C]citrulline would be co-eluted with [^{14}C]urea in the described Dowex chromatography if both were present in the sample. However, we were not able to detect [^{14}C]urea in the citrulline fraction, as no measurable amount of $^{14}CO_2$ was produced in the presence of urease. This is due to the following reasons. First the [^{14}C]arginine formed from [^{14}C]citrulline was diluted by the large amounts of added unlabelled arginine in the incubation medium, minimizing the conversion of [^{14}C]arginine into [^{14}C]urea via arginase. Second, macrophages were separated from the incubation medium before the cells were used for measurements of the intracellular specific radioactivity of [^{14}C]citrulline, therefore effectively removing the small amount of [^{14}C]urea present in the incubation medium.

Uptake of L-citrulline and L-arginine by macrophages

This was measured at 37 °C over a 5 min incubation period in 0.2 ml of oxygenated (O_2/CO_2, 19:1) KHB buffer (pH 7.4) supplemented with 20 mM Hepes, 5 mM glucose and 1 % BSA. The medium also contained 0.1 mM L-[*ureido*-^{14}C]citrulline (1 μCi/μmol) plus 0.5 mM L-arginine or 0.5 mM L-[2,3-^{3}H]-arginine (0.5 μCi/μmol) plus 0.1 mM L-citrulline, respectively, for determination of the uptake of L-citrulline or L-arginine. Incubation media and cell suspensions were warmed up at 37 °C for 5 min, before addition of cells to initiate the measurement of the uptake of [^{14}C]citrulline or [^{3}H]arginine. The uptake of radiolabelled L-citrulline or L-arginine was terminated by addition of 200 μl of ice-cold 10 mM L-citrulline plus D-[2-^{3}H]mannitol (0.1 μCi/ml) or 10 mM L-arginine plus D-[1-^{14}C]mannitol (0.05 μCi/ml) (stop solutions), respectively. D-Mannitol has been used as a suitable extracellular marker for macrophages [21]. The incubation media containing labelled amino acids and mannitol were immediately mixed and transferred to a 1.6 ml microcentrifuge tube, which contained 0.7 ml of a mixture of oil (bromododecane/dodecane, 20:1, v/v) overlaid on 0.2 ml of 1.5 M $HClO_4$ (acid layer). Cells were separated from the medium through the oil layer into the acid layer by centrifugation (10000 *g*, 1 min). The oil layer was washed three times with fresh KHB buffer, and the acid layer containing [^{14}C]citrulline or [^{3}H]arginine was counted for radioactivity in a Beckman liquid-scintillation counter. The amounts of radioactivity of [^{14}C]citrulline or [^{3}H]arginine in the acid layer after correction for the minimal contamination by the incubation medium were used to calculate the uptake of L-citrulline or L-arginine respectively. It was established in preliminary experiments that the uptake of citrulline or arginine by BBn, BBdp and BBd macrophages was linear during a 5 min incubation period.

From the termination of the incubation with the stop solutions to the transfer of the cell suspension to the oil layer for separation, it took 15 s. To assess if the amounts of cellular [^{14}C]citrulline or [^{3}H]arginine were changed after the stop solutions were added to the incubation medium, the cell suspension was either immediately transferred to the oil layer for separation as described above or was left for 15 s at room temperature before transfer to the oil layer for separation into the acid layer. We found that there was no difference in the amounts of [^{14}C]citrulline or [^{3}H]arginine in the acid layer between the above two treatments. This indicates that the use of the stop solutions did not alter the amounts of [^{14}C]citrulline or [^{3}H]arginine present in the cells at the end of the 5 min incubation period, under the present experimental conditions used. Our data suggest that the methods employed in this study were suitable for measuring the uptake of L-citrulline and L-arginine by macrophages.

Statistical analysis

Data were analysed by unpaired *t* test, or one-way analysis of variance with the Student–Newman–Keuls multiple range test [22] as indicated in Tables. *P* values less than 0.05 were taken to indicate statistical significance.

RESULTS

Nitrate production by macrophages

The production of NO_3^- (the major stable oxidation product of NO; [4]) by BBn, BBdp and BBd rat macrophages is shown in Table 1. The rates of production of nitrate increased ($P < 0.01$) by 112 % and 151 % in 75-day-old BBdp and 115-day-old BBd macrophages respectively, compared with age-matched BBn cells. Nitrate production also increased ($P < 0.05$) by 120 % ($n = 3$) in acute (1 day post onset) diabetic BB rats with hyperglycaemia (12.5 ± 2.5 mM serum glucose), compared with age-matched BBn rats (results not shown). There was no difference ($P > 0.05$) in macrophage NO_3^- production between 50-day-old BBdp and BBn rats. No measurable amount of NO_2^- was produced by macrophages during a 2 h incubation period in the presence of 0.5 mM L-arginine. Likewise, in the absence of L-arginine from the incubation medium, no measurable amount of NO_3^- or NO_2^- was generated by BBn, BBdp and BBBd macrophages during the 2 h incubation period.

Conversion of L-citrulline into L-arginine in macrophages

The conversion of L-citrulline into L-arginine in BBdp, BBd and BBn macrophages is presented in Table 1. Unlike NO_3^- production, the L-arginine synthesis from L-citrulline in young (50 days old) BBdp rat macrophages increased ($P < 0.01$) by 704 %, compared with age-matched BBn cells. The rates of L-arginine synthesis from citrulline in macrophages increased ($P < 0.01$) by 892 % and 904 % in 75-day-old BBdp and 115-day-old BBd rats respectively, compared with the cells from age-matched BBn rats. The L-arginine synthesis from L-citrulline also increased ($P < 0.01$) by 820 % ($n = 3$) in acutely (1 day post onset) diabetic BB rats with hyperglycaemia (12.5 ± 2.5 mM serum glucose), compared with age-matched BBn rats (results not shown).

The specific radioactivity of intracellular [^{14}C]citrulline was slightly but significantly ($P < 0.05$) lower than that of [^{14}C]citrulline in the initial incubation medium (Table 2). The specific radioactivity of intracellular [^{14}C]citrulline increased rapidly during the first 0.5 h incubation period and remained fairly constant throughout the remaining incubation period, in BBn, BBdp, and BBd macrophages. There was no difference ($P > 0.05$) in the specific radioactivity of intracellular [^{14}C]-citrulline over the 2 h incubation period between BBdp or BBd macrophages and age-matched BBn cells.

Percentage recycling of L-citrulline into L-arginine in macrophages

The percentage recycling of L-citrulline into L-arginine in macrophages was estimated [15] on the basis of the fact that 1 mol of L-arginine is stoichiometrically converted into 1 mol each of NO

Table 1 Nitrate production from L-arginine and conversion of L-citrulline into L-arginine in rat macrophages

Macrophages were incubated at 37 °C for 2 h in the presence of 0.5 mM L-arginine plus 0.1 mM L-[*ureido*-^{14}C]citrulline as described in the text. The percentage of L-citrulline recycled into L-arginine was estimated on the basis of NO_3^- production from L-arginine and the conversion of L-citrulline into L-arginine. Data are means ± S.E.M. for the numbers of animals in parentheses. *$P < 0.01$: significantly different from age-matched BBn rats as analysed by unpaired *t* test.

Rats	NO_3^- production (nmol/2 h per 10^6 cells)	[^{14}C]Arginine synthesis from [^{14}C]citrulline (nmol/2 h per 10^6 cells)	L-Citrulline recycled into L-arginine (%)
50-day-old BBn (8)	2.30 ± 0.13	0.23 ± 0.03	10.3 ± 1.4
50-day-old BBdp (8)	2.61 ± 0.21	1.85 ± 0.26*	70.5 ± 6.8*
75-day-old BBn (7)	2.40 ± 0.23	0.25 ± 0.04	11.7 ± 2.1
75-day-old BBdp (7)	5.09 ± 0.52*	2.48 ± 0.30*	51.6 ± 8.9*
115-day-old BBn (11)	2.33 ± 0.28	0.26 ± 0.02	11.9 ± 1.4
115-day-old BBd (11)	5.85 ± 0.52*	2.61 ± 0.19*	44.2 ± 2.9*

Table 2 Specific radioactivity of intracellular [^{14}C]citrulline in rat macrophages

Macrophages were incubated for 0.5–2 h in the presence of 0.1 mM L-[*ureido*-^{14}C]citrulline plus 0.5 mM L-arginine. The specific radioactivity of [^{14}C]citrulline in the initial incubation medium was 1958±29 d.p.m./nmol. All data are means±S.E.M. ($n = 4$). There was no difference ($P > 0.05$) in the sp. radioactivity of intracellular [^{14}C]citrulline among the different time points within each row, as analysed by one-way analysis of variance.

	Sp. radioactivity (d.p.m./nmol)		
Rats Incubation period...	0.5 h	1.0 h	2.0 h
75-day-old BBn	1747±42	1789±50	1717±35
75-day-old BBdp	1756±25	1820±22	1876±54
115-day-old BBn	1723±41	1776±35	1749±48
115-day-old BBd	1692±36	1748±42	1765±46

Table 3 Uptake of L-citrulline and L-arginine by rat macrophages

Macrophages were incubated at 37 °C for 5 min in the presence of 0.1 mM L-[^{14}C]citrulline plus 0.5 mM L-arginine to measure L-citrulline uptake, or in the presence of 0.5 mM L-[^{3}H]arginine plus 0.1 mM L-citrulline to measure L-arginine uptake, as described in the text. Data are means±S.E.M. ($n = 6$). *$P < 0.01$: Significantly different from age-matched BBn rats as analysed by unpaired t test.

	Uptake (pmol/min per 10^6 cells)	
Rats	L-Citrulline	L-Arginine
50-day-old BBn	21.2±2.6	88.3±8.5
50-day-old BBdp	22.6±2.2	102.4±8.0
75-day-old BBn	23.9±1.3	103.1±6.1
75-day-old BBdp	25.8±1.9	139.2±4.3*
115-day-old BBn	24.2±1.8	108.0±4.5
115-day-old BBdp	27.6±2.1	134.8±6.4*

Table 4 Percentage of L-citrulline or L-arginine taken up by rat macrophages that is converted into L-arginine or NO respectively

Data are means±S.E.M. for the numbers of animals in parentheses. The percentage of the L-citrulline taken up by macrophages that is converted into L-arginine was calculated on the basis of L-citrulline uptake and L-arginine synthesis. Similarly, the percentage of the L-arginine taken up by macrophages that is converted into NO was calculated on the basis of L-arginine uptake and NO_3^- production. *$P < 0.01$: significantly different from age-matched BBn rats as analysed by unpaired t test.

Rats	Percentage of L-citrulline taken up by macrophages that is converted into L-arginine	Percentage of L-arginine taken up by macrophages that is converted into NO
50-day-old BBn (8)	9.2±1.1	20.9±1.2
50-day-old BBdp (8)	64.0±6.3*	21.2±1.7
75-day-old BBn (6)	8.7±1.5	19.4±1.9
75-day-old BBdp (6)	70.2±6.6*	30.7±3.2*
115-day-old BBn (11)	10.5±1.4	18.1±1.7
115-day-old BBdp (11)	68.4±5.2*	36.1±3.2*

and L-citrulline [23], and is presented in Table 1. About 10–12% of L-citrulline could be recycled into L-arginine in macrophages from 50–115-day-old BBn rats. The rates of citrulline recycling into arginine were markedly increased ($P < 0.01$), by 7-, 5- and 4-fold in macrophages from 50-day-old BBdp, 75-day-old BBdp and 115-day-old BBd rats respectively, compared with cells from age-matched BBn rats.

Uptake of L-citrulline by macrophages

The uptake of L-citrulline by BBdp, BBd and BBn macrophages is shown in Table 3. There were no differences ($P > 0.05$) in the rates of L-citrulline uptake by macrophages between 50-day-old BBdp, 75-day-old BBdp or 115-day-old BBd rats and age-matched BBn rats. The percentage of the L-citrulline taken up by macrophages that was converted into L-arginine was about 10% in BBn rats, and markedly increased ($P < 0.01$) to 60–70% in BBdp and BBd rats (Table 4). It is noteworthy that the rates of L-citrulline uptake by macrophages exceeded those of the conversion of L-citrulline into L-arginine in the cells from BBn, BBdp and BBd rats.

Since [^{14}C]citrulline can be converted into [^{14}C]arginine in macrophages, which may be exported into the medium, measurement of intracellular ^{14}C radioactivity would underestimate L-citrulline uptake by the cells. We found that the amount of [^{14}C]citrulline-derived [^{14}C]arginine accumulated in the medium during a 5 min incubation period was 6.2±1.3 and 7.5±0.8% (mean±S.E.M., $n = 5$) of intracellular ^{14}C radioactivity in BBn and BBd macrophages respectively. Thus the measurement of intracellular ^{14}C radioactivity would underestimate L-citrulline uptake by macrophages by less than 8% under the experimental conditions used.

Uptake of L-arginine by macrophages

The accumulation of [^{3}H]arginine in macrophages is used as an index of L-arginine uptake [23], and is presented in Table 3. The rates of L-arginine uptake increased ($P < 0.05$) by 35% and 25% in 75-day-old BBdp and 115-day-old BBd macrophages respectively, compared with age-matched BBn cells. However, there was no difference ($P > 0.05$) in L-arginine uptake by macrophages between younger (50-day-old) BBdp and BBn rats. The percentage of the L-arginine taken up by macrophages that was converted into NO was about 20% in BBn rats, and increased ($P < 0.01$) to 30–36% in 75-day-old BBdp and 115-day-old BBd rats (Table 4). It is noteworthy that the rates of L-arginine uptake by macrophages exceeded those of NO_3^- production by the cells from BBn, BBdp and BBd rats.

DISCUSSION

We have demonstrated for the first time the activation of the arginine–citrulline cycle in macrophages in such an autoimmune condition as spontaneous immunologically mediated diabetes in the BB rat (Table 1). The rate of the increased NO synthesis in 75-day-old BBdp and BBd rat macrophages was similar to that reported for lipopolysaccharide-activated rat macrophages [15]. It is noteworthy that the rates of L-arginine synthesis from L-citrulline increased by 3-fold in lipopolysaccharide-activated macrophages [15], but by 7–9 fold in BBdp and BBd cells (Table 1). The increased conversion of L-citrulline into L-arginine measured with L-[*ureido*-^{14}C]citrulline was unlikely to be due to dilution of the labelled precursor, as the intracellular specific radioactivity of intracellular [^{14}C]citrulline was similar between BBdp or BBd macrophages and age-matched BBn cells (Table 2).

The increased activity of the macrophage arginine–citrulline cycle occurred in the BBdp rats and the insulin-treated BBd rats with normal blood glucose concentrations and in the acutely

diabetic BBd rats with hyperglycaemia and hypoinsulinaemia (see the Results section). This suggests that blood concentrations of glucose and insulin may not be primary factors responsible for the increased L-citrulline recycling into L-arginine and NO synthesis in BBdp and BBd macrophages. Although the precise mechanisms for the increased arginine–citrulline-cycle activity have not been elucidated in BBdp and BBd macrophages, some possibilities can be suggested on the basis of the present findings. We demonstrated that the rate of uptake of L-citrulline by macrophages exceeded that of the conversion of L-citrulline into L-arginine (Table 4). The rapid uptake of L-citrulline by macrophages was consistent with our measurements of the specific radioactivity of intracellular [^{14}C]citrulline, which rapidly reached a steady state within a 0.5 h incubation period (Table 2). Thus the uptake of L-citrulline by macrophages would not be expected to be a limiting factor for the synthesis of L-arginine from extracellular L-citrulline. This suggestion is borne out by our findings that there was no difference in L-citrulline uptake by macrophages between BBdp or BBd rats and age-matched BBn rats (Table 3). Although the uptake of L-arginine by macrophages increased by 25–35% in 75-day-old BBdp and 115-day-old BBd rats compared with age-matched BBn rats, an increase of this magnitude would not fully account for the 110–150% increase in NO synthesis by the cells from these BBdp and BBd rats (Table 1). Thus we suggest that the increased activity of the arginine–citrulline cycle may primarily be due to the activation of the enzymes involved, rather than the uptake of substrates. This suggestion has not been tested in the present study, because of the limited supply of a large number of expensive BB rats required for obtaining adequate amounts of peritoneal macrophages for assays of enzyme activities.

The pathways of L-arginine synthesis from L-citrulline and of NO generation from L-arginine in macrophages appear to be differentially activated in the spontaneously diabetic BB rat. For example, the conversion of L-citrulline into L-arginine increased by 704% in 50-day-old BBdp rat macrophages, whereas there was no difference in macrophage NO synthesis between 50-day-old BBdp and BBn rats (Table 1). This implies that an increase in intracellular L-arginine synthesis from L-citrulline may be independent of NO synthesis, when NO synthase is not activated. It is probable that the persistent elevation in the recycling of L-citrulline into L-arginine in BBdp and BBd macrophages may reflect an innate metabolic disorder in these cells. On the other hand, the enhanced NO synthesis in older BBdp and BBd rat macrophages may evolve as a result of the process of the spontaneous autoimmune activation of macrophages.

The pancreatic islets in BBdp rats are intensively infiltrated by mononuclear cells, including activated macrophages, 2–3 weeks before the onset of overt diabetes [24,25]. We recognize that the macrophages infiltrating the pancreas, rather than those present in the peritoneum, are directly involved in the destruction of β-cells in the spontaneously diabetic BB rat. However, peritoneal macrophages were used in this study, mainly because it is very difficult to obtain adequate amounts of homogeneous macrophages from rat pancreas for metabolic studies and because macrophages prepared from different anatomical locations have similar metabolic patterns and functions [6,16,18,26–28]. Assuming that the macrophages infiltrating the islets have an activated arginine–citrulline cycle, as do peritoneal macrophages, the increased macrophage L-arginine synthesis from L-citrulline (Table 1) may have significant implications in the BBdp rat. It is well established that the extracellular space of inflammatory sites contains an exceedingly low concentration of L-arginine, owing to the high activity of macrophage-derived arginase [28], which converts L-arginine into ornithine and urea. Thus the increased intracellular recycling of L-citrulline into L-arginine in BBdp and BBd macrophages may be important for the sustained provision of L-arginine for NO synthesis by these cells in inflammatory sites such as the pancreatic islets infiltrated by mononuclear cells.

NO is highly reactive with iron and thiols, thereby modulating the function of proteins and accounting for its cytotoxicity [29]. Recently, macrophages have been implicated in the pathogenesis of autoimmune insulitis and diabetes via NO generation [7,10]. Consistent with this hypothesis are our findings that NO synthesis was markedly increased in macrophages from 75-day-old BBdp and 115-day-old BBd rats whose pancreatic islets have been shown to be intensely infiltrated by macrophages and lymphocytes [24,25]. It is noteworthy that the elevated NO synthesis by macrophages from 75-day-old BBdp and 115-day-old BBd rats coincides with an increase in glucose metabolism via the pentose phosphate pathway [16,17] to provide NADPH for NO synthesis in these cells. NO may destroy pancreatic β-cells either directly and/or by acting synergistically with its reactive derivatives. A recent study has reported that peroxynitrite ($OONO^-$), which is rapidly generated from either NO and O_2^- [30] or NO^+ (a redox form of NO) and H_2O_2 [29] in an aqueous solution, is a highly potent oxidant mediating the oxidation of both non-protein and protein thiols [31]. An enhanced cytotoxic potential of $OONO^-$ may be achieved when the rates of production of NO, O_2^- and H_2O_2 are increased. Interestingly, we have recently demonstrated enhanced production of O_2^- and H_2O_2 in 75-day-old BBdp and 115-day-old BBd rat macrophages [17]. Once attracted into pancreatic islets due to β-cell-specific antigenic changes [32], or for other as yet unidentified reasons, activated macrophages may release large amounts of NO (Table 1), O_2^- and H_2O_2 [17]. The increase in NO synthesis in BBdp and BBd macrophages may be sustained by maintaining a sufficient intracellular L-arginine concentration via the recycling of L-citrulline into L-arginine (Table 1), when the cells are present in the inflammatory sites of the pancreatic islets. It is possible that an inhibition of NO synthesis in BBdp rat macrophages may preclude autoimmune destruction of pancreatic β-cells. In this regard, it is noteworthy that cyclosporins, which can prevent diabetes in the BB rat (e.g. [33]), have recently been reported to inhibit NO synthesis in macrophages [34]. Thus, in light of the recent advances in understanding the biology of NO [1,2] and the increased NO synthesis in BBdp and BBd rat macrophages (Table 1), a role for NO in β-cell destruction in the BB rat warrants detailed investigations.

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