Decrease in free-radical production with age in rat peritoneal macrophages

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The respiratory-burst reaction has been studied in rat peritoneal macrophages of different ages (3, 12 and 24 months) using phorbol 12-myristate 13-acetate (PMA) to stimulate NADPH oxidase. Production of $O_2^{-\cdot}$ and H_2O_2 decreased with age (about 50 and 75% respectively); however, no difference in NADPH oxidase activity was found. NO· production was also reduced with age (40%). Furthermore, a progressive and significant decrease in the pentose phosphate flux was detected as a function of age in control and PMA-stimulated macrophages. The NADPH/$NADP^+$ ratio decreased with age in control and PMA-stimulated macrophages. Glucose uptake was lower in middle-aged (12 months) and old (24 months) animals but no differences were found between these groups.

INTRODUCTION

Immune function declines with age [1]. The impairment in most T- and B-cell functions is well established [2,3]. However, the effects of aging on macrophages, which participate in a variety of host defence mechanisms, are quite contradictory. There are reports that macrophage-dependent host defence mechanisms are decreased [4–6], apparently unaffected [7] or even enhanced [8] with age.

Macrophages are the most important phagocytic cells. Activated macrophages kill micro-organisms, tumour cells and damaged tissue during inflammation by two separate oxidative pathways involving the synthesis of reactive oxygen species [9] and NO· [10]. Reactive oxygen species are produced in a metabolic pathway called the respiratory burst. The respiratory burst consists of a sudden and marked activation of oxidative metabolism, resulting in the production of $O_2^{-\cdot}$ and derived toxic species such as H_2O_2. This mechanism is induced in macrophages by exposure to appropriate stimuli which activate the $O_2^{-\cdot}$-generating enzyme called NADPH oxidase [11]. NO· is produced by oxidizing one of the equivalent guanidinium nitrogens of L-arginine [12]. The formation of NO· and reactive oxygen species requires NADPH as reducing agent. A relationship exists between the NADPH required in the respiratory burst, NO· production and the pentose phosphate pathway [13,14]. It has been suggested that 'malic' enzyme may also produce NADPH in macrophages [15].

We have recently produced evidence that the respiratory-burst reaction, determined as $O_2^{-\cdot}$ production, decreases with age in rat peritoneal macrophages [16]. We have also found a decrease with age in the activity of the two most important enzymes of the pentose phosphate shunt; glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

In the present work we extend these observations to other parameters (namely H_2O_2 production and NADPH oxidase activity). In addition, we have identified other metabolic parameters (NO· production, pentose phosphate flux, NADPH and $NADP^+$ levels and glucose transport) that are altered with age.

MATERIALS AND METHODS

Animals

Young (3 months), mature (12 months) and old (24 months) female Wistar rats were used. They were maintained on a standard laboratory diet with free access to food and water.

Chemicals

Chemicals were of analytical grade from Merck (Barcelona, Spain). Biochemical reagents and enzymes were from Boehringer-Mannheim (Barcelona, Spain) or from Sigma Chemical Co. (Alcobendas, Spain). [1-^{14}C]Glucose, [6-^{14}C]glucose and 2-deoxy-D-[U-^{14}C]glucose (2dGlc) were obtained from Amersham International (Amersham, Bucks., U.K.). Culture media were from ICN (Barcelona, Spain).

Stock solutions of phorbol 12-myristate 13-acetate (PMA) were dissolved at 1 mg/ml in DMSO and stored at −20 °C. Scopoletin (7-hydroxy-6-methoxycoumarin) was dissolved in 0.5 M phosphate buffer (pH 7.0) to a concentration of 0.2 mM. Peroxidase was prepared in water at 5 mg/ml and stored at −20 °C until needed.

Preparation of peritoneal macrophages

Peritoneal macrophages were obtained from Wistar rats by the method of Tsunawski and Nathan [17]. Female Wistar rats were injected intraperitoneally 4 days before harvest with 5 ml of 6% sodium caseinate. They were killed by decapitation and the peritoneal cavity was immediately washed with 10 ml of saline solution. Cells were pelleted by centrifugation, resuspended in Krebs–Ringer bicarbonate buffer (KRB) and immediately used for experiments. Viability, as determined by Trypan Blue exclusion, was always higher than 95%.

Assay of $O_2^{-\cdot}$ and H_2O_2

Cells (1×10^6–1.5×10^6 cells/ml in oxygenated KRB with 5, 10, 15 and 20 mM glucose) were prewarmed at 37 °C with 80 μM

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; 2dGlc, 2-deoxy-D-glucose; KRB, Krebs–Ringer bicarbonate buffer.

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cytochrome *c* for 5 min before addition of PMA. Cytochrome *c* reduction was recorded continuously at 550 nm using a spectrophotometer. $O_2^{-\cdot}$ production was estimated by measuring the superoxide dismutase-inhibitable reduction of cytochrome *c* at 37 °C, as previously described [18], using a molar absorption coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$.

H_2O_2 was determined using the scopoletin assay [19]. Oxidation of scopoletin by H_2O_2 was followed fluorimetrically at 37 °C at an excitation wavelength of 366 nm and an emission wavelength of 466 nm. The reaction mixture contained 2 μM scopoletin, 1 mM NaN_3, 45 units of horseradish peroxidase/ml, 10 mM glucose, 100 nM PMA and 1×10^6–1.5×10^6 cells/ml. To calculate the amount of H_2O_2 produced, a standard curve was constructed by measuring the fluorescence of solutions containing 45 units/ml peroxidase, 2 μM scopoletin and 5, 10, 20 and 30 μl of standard H_2O_2 solution (final volume 1.0 ml).

Macrophage culture

Macrophages were isolated by the general method. The cells were resuspended in culture medium (RPMI 1640, 20 mM Hepes, 2 mM L-glutamine, 10 % fetal calf serum and antibiotics), plated (5×10^5 cells/300 μl) in flat-bottomed 96-well culture plates and incubated at 37 °C for 2 h in 95 % O_2/5 % CO_2. Non-adherent cells were removed by washing three times with RPMI 1640, and macrophage monolayers were then cultured in culture medium for 72 h.

Nitrite production

$NO^{\cdot}$ release from macrophages, after incubation for 72 h, was assessed by determining the nitrite concentration in the culture supernatant using the Griess reaction [20] as used by others [21,22]. Nitrite concentration was calculated from an $NaNO_2$ standard curve.

Assay of NADPH oxidase

Macrophages were isolated by the general method. They were prewarmed in KRB with 10 mM glucose at 37 °C for 3 min. PMA (100 nM) (or DMSO in the controls) prewarmed at 37 °C for 5 min was added, and the reaction was stopped by putting in ice. Centrifugation was carried out at 400 *g* for 5 min and the resultant pellet was resuspended in 0.34 M sucrose. The cells were then sonicated at 4 °C (3–4 × 10 s). Cell disruption was monitored using the Trypan Blue-exclusion method. Centrifugation was carried out at 800 *g* for 10 min and the supernatant used to determine enzyme activity. NADPH oxidase activity was determined spectrophotometrically by measuring cytochrome *c* reduction at 550 nm. The reaction mixture contained 10 mM phosphate buffer (pH 7.2), 100 mM NaCl, 1 mM $MgCl_2$, 80 μM cytochrome *c*, 2 mM NaN_3 and 100 μl of supernatant (final volume 1.0 ml). A suitable amount of NADPH (10–20 μl) was added last to initiate the reaction [23].

Measurement of nicotinamide nucleotides

The concentrations of $NADP^+$ and NADPH in peritoneal macrophages were estimated essentially as described by Greenbaum et al. [24]. Incubations were stopped by adding to 1 ml of incubation medium either 0.1 ml of 2 M KOH for NADPH determinations or 0.1 ml of 20 % $HClO_4$ for $NADP^+$ determinations. Both extracts were incubated at 60 °C for 10 min and neutralized, and the soluble supernatants were used for $NADP^+$ or NADPH determination. The reaction mixture was held in the reaction chamber of a Clark-type electrode and contained, in a volume of 1 ml, 0.15 M Tris/HCl, pH 8.0, 1.2 mM glucose 6-phosphate, 0.6 mM EDTA, 0.8 mM phenazine methosulphate, 10 units of glucose-6-phosphate dehydrogenase and different amounts of acid or alkaline extracts. The concentration of both nucleotides was measured as the rate of O_2 consumption in the mixture after the addition of glucose-6-phosphate dehydrogenase. An internal standard of $NADP^+$ or NADPH was used for each assay.

Activity of the hexose monophosphate shunt

[1-^{14}C]Glucose and [6-^{14}C]glucose were kept frozen at −20 °C to a specific radioactivity of 50 μCi/250 μl. A working solution was prepared by diluting the stock solution with 10 mM glucose (final radioisotope concentration 0.1 μCi/tube). Macrophages (10×10^6 cells/ml) were added to KRB containing 1 mM glucose. $^{14}CO_2$ released was measured by collecting it in 0.2 ml of hyamine impregnated in Whatman filter papers placed in the centre of an Erlenmeyer flask. The radioactivity recovered in hyamine was determined. Hexose monophosphate shunt activity was determined by subtracting the counts obtained from the oxidation of [6-^{14}C]glucose from those obtained from the oxidation of [1-^{14}C]glucose [25].

Determination of glucose uptake

Transport of 2-deoxy-D-glucose in rat peritoneal macrophages was investigated as described by Rist et al. [26] with minor modifications. Briefly, macrophages (10×10^6 cells/ml) were added to KRB containing 0.1 mM 2dGlc at a final radioisotope concentration of 0.1 μCi/tube). After incubation, sugar uptake was halted by high-speed centrifugation for 30 s. The supernatants were removed, and the cells washed twice by adding iso-osmotic ice-cold solution containing 1 μm cytochalasin B and 1 μM $HgCl_2$ and re-centrifuged to remove extracellular radioisotope. Finally, the cell pellets were resuspended in Triton X-100 and placed in scintillation fluid to determine the total uptake. The rates of glucose uptake were determined from 5 min to 3 h.

Statistical methods

Student's *t* test was utilized to evaluate differences between means, and the 0.05 level of probability was used as the criterion of significance. All values reported are means ± S.D. of experiments on at least five animals.

RESULTS

Effect of age on respiratory-burst reaction

Respiratory burst is induced in phagocytic cells by exposure to appropriate stimuli. The tumour promoter, PMA, is reported to be the most potent stimulatory agent of respiratory burst [27]. We have analysed $O_2^{-\cdot}$ production in PMA-stimulated peritoneal macrophages from rats of different ages at several glucose concentrations (5, 10, 15 and 20 mM). Figure 1 demonstrates that, at all concentrations studied, there were differences between the three groups (3-, 12- and 24-month-old rats). At all ages the maximal response was at 10 mM glucose. These results extend previous observations [16] and show that increasing glucose concentration does not improve the age-related impairment of the respiratory burst in either middle-aged or old animals.

The decrease in $O_2^{-\cdot}$ production with age may be due to greater dismutation in middle-aged and old animals than in

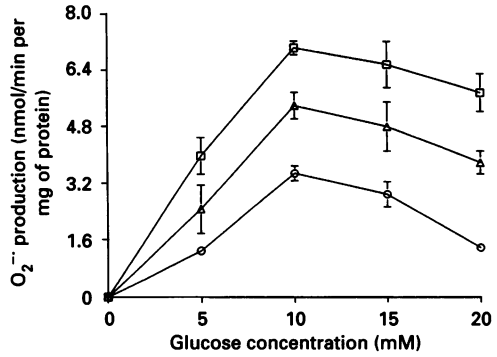

Figure 1 $O_2^{-\cdot}$ production by PMA-induced peritoneal macrophages at different glucose concentrations

Peritoneal macrophages from rats of 3 months (□), 12 months (△) and 24 months (○) of age were incubated with 100 nM PMA. $O_2^{-\cdot}$ production was measured as described in the Materials and methods section. Data are means ± S.D. from at least five separate experiments. Results from the three groups were statistically significant at all the glucose concentrations studied.

young ones. Thus further experiments were performed to analyse H_2O_2 production. We found that peritoneal macrophages induced by PMA display a progressive reduction in their capacity to produce H_2O_2 with increasing age (Figure 2). The decrease was about 50 and 75% in middle-aged and old animals respectively.

Another possible explanation is the NADPH oxidase activity is altered with age. Although production of $O_2^{-\cdot}$ and H_2O_2 decreases with age, no difference was found in NADPH oxidase activity. The values were 3.61 ± 0.41 (3-month-old rats), 3.54 ± 0.34 (12-month-old rats) and 3.73 ± 0.27 nmol of $O_2^{-\cdot}$/min per mg of protein (24-month-old rats).

Effect of age on nitrite production

Nitrite production was measured in cultured macrophages from rats of different ages. As shown in Figure 3, a highly significant decrease was found between 3 and 24 months of age ($P < 0.001$). The decrease between 3- and 12-month-old rats was also significant ($P < 0.01$); however, there was a slight but not significant diminution between 12- and 24-month-old rats.

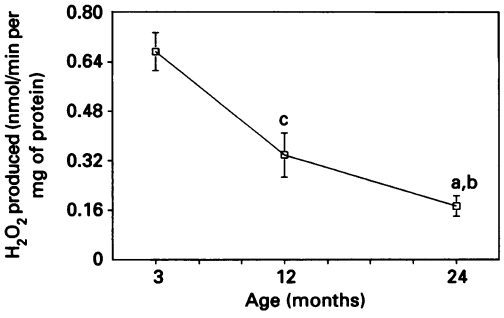

Figure 2 H_2O_2 release by PMA-induced peritoneal macrophages

Peritoneal macrophages from rats of different ages were incubated with 100 nM PMA. H_2O_2 production was measured as described in the Materials and methods section. Data are means ± S.D. from at least five separate experiments. Statistical significance: a (differences between rats aged 24 and 3 months) $P < 0.001$; b (differences between rats aged 24 and 12 months) $P < 0.005$; c (difference between rats aged 12 and 3 months) $P < 0.001$.

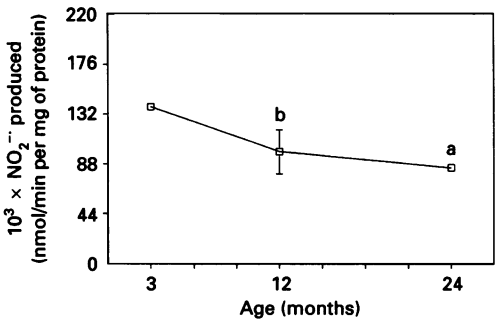

Figure 3 Nitrite production by peritoneal macrophages

Nitrite production was measured as described in the Materials and methods section. Data are means ± S.D. from at least five separate experiments. Statistical significance: a (differences between rats aged 24 and 3 months) $P < 0.001$; b (differences between rats aged 12 and 3 months) $P < 0.01$.

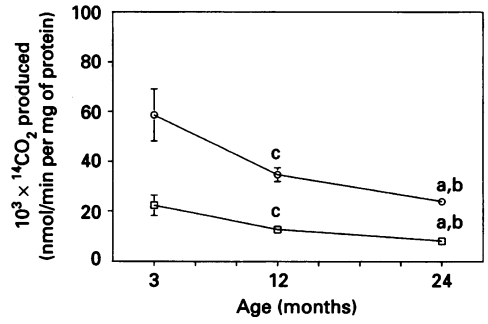

Figure 4 Pentose phosphate pathway activity in control (□) and PMA-stimulated (○) peritoneal macrophages

Flux through the pentose phosphate pathway was measured as described in the Materials and methods section. Data are means ± S.D. from at least five separate experiments. Statistical significance: a (difference between rats aged 24 and 3 months) $P < 0.02$; b (difference between rats aged 24 and 12 months) $P < 0.01$; c (difference between rats aged 12 and 3 months) $P < 0.01$.

Effect of age on activity of the pentose phosphate pathway and glucose transport

In macrophages, there is a significant pentose phosphate pathway [13]. In order to explain the reduced capacity of macrophages from old rats to produce $O_2^{-\cdot}$, we studied this pathway. Figure 4 shows a progressive and significant age-related decrease in pentose phosphate flux; the decline was about 64%. The values were considerably increased when the macrophages were incubated with 100 nM PMA. The age-related decrease in the stimulated macrophages was about 60%.

Recently a close relationship between pentose phosphate flux and glucose transport has been shown in macrophages [26]. The accumulation of free 2dGlc in rat macrophages is phorbol-independent [26]. There was a progressive and linear increase in glucose uptake in rat peritoneal macrophages for about the first

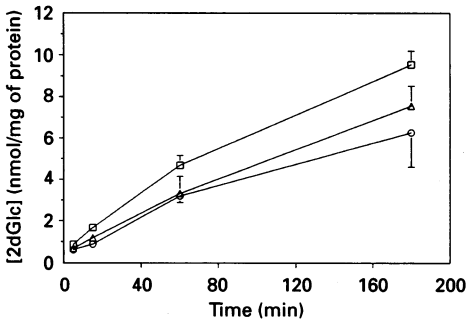

Figure 5 Uptake of 2dGlc into rat peritoneal macrophages

Peritoneal macrophages from rats aged 3 (□), 12 (△) and 24 (○) months were obtained and glucose uptake was measured as described in the Materials and methods section. Data are means ± S.D. from at least five separate experiments. Results from rats aged 3 months were statistically significantly different from those aged 12 and 24 months at all the times studied. Differences between rats aged 12 and 24 months were not significant at any time.

60 min at all ages studied (Figure 5). We found statistically significant differences between rats aged 3 months and those aged 12 or 24 months at all the times assayed. The differences between rats aged 12 and 24 months were not statistically significant at any time studied.

Effect of age on $NADP^+$ and NADPH concentrations

In control peritoneal macrophages to the NADPH content decreased with age (Table 1). A significant decrease was found between 3 and 24 months ($P < 0.025$). The decrease between 3- and 12-month-old rats was also significant ($P < 0.05$), but the differences between 12- and 24-month-old rats was not significant. $NADP^+$ levels were higher in old and middle-aged rats than in young animals. The NADPH/$NADP^+$ ratio decreased from 1.60 ± 0.05 nmol/mg in 3-month-old rats to 1.22 ± 0.05 nmol/mg in 24-month-old animals.

In PMA-stimulated peritoneal macrophages the NADPH/NADP ratio was lower in all ages than in control macrophages. This is due to NADPH consumption during the respiratory burst. The NADPH levels decreased with age and $NADP^+$ levels increased, so we found a significant decrease in the NADPH/NADP ratio between the three groups studied.

DISCUSSION

Free-radical production by many tissues seems to increase with age, this being the main theory of aging [28]. In contrast, however, free-radical production by rat peritoneal macrophages seems to decrease with age, although this fact is controversial [5–8,29].

Recent reports from our laboratory using various stimuli (PMA, concanavalin A and *N*-formylmethionyl-leucylphenylalanine) have shown that aging reduces $O_2^{-\bullet}$ production in macrophages [16]. The greatest decrease (about 50%) was observed with PMA (the best stimulant respiratory burst described). Ganguly et al. [6] have also described a decline in $O_2^{-\bullet}$ production by rat alveolar macrophages from aged animals stimulated with opsonized zymosan.

As the respiratory burst depends on glucose availability, in the present work we tested whether an increase in glucose concentration would increase the respiratory-burst reaction. As shown in Figure 1, at all ages studied maximum values were obtained with 10 mM glucose, the concentration used in our previous studies [16], and with higher concentrations $O_2^{-\bullet}$ production decreased. Chiara et al. [30] reported maximal response using 5 and 10 mM glucose in young rats. Other authors have demonstrated a large decrease in the respiratory burst with high glucose concentrations, probably the result of a glycosylation process [31].

The decline in $O_2^{-\bullet}$ production (30% in middle-aged and 50% in old rats with 10 mM glucose) could be due to a major dismutation, with formation of H_2O_2. However, we found an even more pronounced decrease in H_2O_2 levels (about 50 and 75% in 12- and 24-month-old animals respectively). Costa Rosa et al. [32] reported that H_2O_2 formation by tumour-activated macrophages was also markedly reduced in aged rats. The decrease in H_2O_2 seems to indicate that the decrease in $O_2^{-\bullet}$ is not due to a major dismutation. The values for H_2O_2 production observed in young animals are approximately the same as previously described [30]. We did not find a good correlation between $O_2^{-\bullet}$ and H_2O_2 levels; similar discrepancies have been described by others [33–35].

As the enzyme involved in the respiratory burst is NADPH oxidase, we investigated whether the age-related dysfunction in production of reactive oxygen could be due to a decrease in NADPH oxidase activity, as has been reported for many enzymes in the elderly [36,37]. We found no differences between the three groups, so the aged-related decline in respiratory burst cannot be attributed to differences in any component of the NADPH oxidase system.

Another mechanism by which macrophages carry out their

Table 1 NADPH and $NADP^+$ concentrations in control and PMA-stimulated peritoneal macrophages from rats of different ages

Data are means ± S.D. from at least five separate experiments and are expressed as nmol/mg of protein. Statistical significance: [a](difference between rats aged 24 and 3 months) $P < 0.025$; [b](difference between rats aged 24 and 12 months) $P < 0.025$; [c](difference between rats aged 12 and 3 months) $P < 0.05$.

Additions to cells	Parameter measured	3 months	12 months	24 months
None	NADPH	2.50 ± 0.07	2.18 ± 0.17^c	2.09 ± 0.09^a
	$NADP^+$	1.56 ± 0.05	1.69 ± 0.06^c	1.71 ± 0.01^a
	NADPH/$NADP^+$	1.60 ± 0.05	1.29 ± 0.09^c	1.22 ± 0.05^a
PMA (100 nM)	NADPH	1.65 ± 0.12	1.44 ± 0.05^c	1.34 ± 0.06^a
	$NADP^+$	2.22 ± 0.11	2.52 ± 0.15^c	2.69 ± 0.09^a
	NADPH/$NADP^+$	0.74 ± 0.09	0.57 ± 0.02^c	$0.49 \pm 0.03^{a,b}$

immunological function is NO• production. Macrophages synthesize NO• and citrulline from L-arginine. NO• may itself inhibit the activity of important metabolic enzymes or form more toxic radicals when combined with $O_2^{-\bullet}$ radicals {e.g. peroxynitrite ($ONOO^-$), which is a much stronger oxidant than NO• [38]}. NO• reacts readily with O_2 to yield NO_2 which ultimately reacts to form $NO_2^{-\bullet}$ and $NO_3^{-\bullet}$ via the hydrolysis of N_2O_3 and N_2O_4 [39]. We found a reduction in nitrite formation with age (Figure 4). The differences were more pronounced between 3 and 12 months, and only a slight decrease was found between 12 and 24 months. A decline in NO• production with age has also been reported in various rat brain regions [40].

The decrease in NO• production may be related to various processes. NO• production appears to be modulated by products of the lipoxygense pathway of arachidonic acid metabolism [41] and we have reported elsewhere [42] a reduction (about 50%) in arachidonic acid content in rat peritoneal macrophage membranes. On the other hand, it has been reported recently that NO• production is linked to the induction of glucose-6-phosphate dehydrogenase [43] and we have reported a reduction in this enzyme activity in macrophages from aged animals [16]. NO• generation from activated macrophages has also been shown to be stimulated by GSH [44]; as we have previously reported a decline in glutathione reductase activity with age [16], the level of GSH in old animals may be lower then in younger ones.

Both nitrogen oxidation products and reactive intermediates of oxygen require the same reducing agent, NADPH. An explanation for the decrease in $O_2^{-\bullet}$ and NO• levels might be that there is a decrease in NADPH levels. This hypothesis is supported by previous assumptions in the literature that relate free-radical production to NADPH formation. Thus Baehner et al. [45] reported that patients with a dramatic reduction in glucose-6-phosphate dehydrogenase activity show a decrease in oxidase activity. A decline in $O_2^{-\bullet}$ production with age has also been associated with a diminution of the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [16]. Other indirect evidence obtained in macrophage studies related to the link between the hexose monophosphate shunt and $O_2^{-\bullet}$ is reviewed by Johnson [46].

We investigated whether a decrease in the hexose monophosphate shunt may contribute to the age-related impairment of $O_2^{-\bullet}$ and NO• production. The data presented in this paper show a decrease with age in the hexose monophosphate shunt both in basal conditions and after PMS stimulation (Figure 4). However, the activity of this pathway was about 100-fold lower than the rate of $O_2^{-\bullet}$ production. In this context, Borregaard et al. [47] also report that the hexose monophosphate shunt accounts for less than 10% of the NADPH used for $O_2^{-\bullet}$ production in neutrophils. Recently, it has been suggested that 'malic' enzyme in addition to the hexose monophosphate shunt can produce NADPH in macrophages [15].

Glucose transport, which is closely linked to hexose monophosphate shunt [19], was reduced in old animals (24 months) relative to young animals (3 months) (Figure 5). Similar results have also been reported for small intestine [48], adipose tissue and skeletal muscle [49], and human [50,51] and rat [52] adipocytes.

NADPH concentration and NADPH/$NADP^+$ ratio were also lower in old and middle-aged rats than young rats (Table 1). Although we have found an age-dependent difference in the values of NADPH and $NADP^+$, they are much less pronounced than age-dependent changes in $O_2^{-\bullet}$ and NO• production. These results appear to exclude a causal relationship between pentose-phosphate-pathway-mediated NADPH synthesis and rate of $O_2^{-\bullet}$ and NO• production.

We have found no clear explanation for the decrease in $O_2^{-\bullet}$ production with age. Other possible explanations for the age-related alteration in the respiratory burst may involve the signal-transduction pathway. A marked reduction in Ca^{2+} mobilization after activation has been reported in neutrophils from elderly people [53]. Also, a defect in cell membrane lipid or phosphoinositol metabolism could be related to the decline in $O_2^{-\bullet}$ production. Indeed, decreases in membrane fluidity and phosphoinositol levels have been described by us in macrophages from aged animals [42].

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