Biochimica et Biophysica Acta, 1145 (1993) 199–204

BBAMEM 75859

Effects of alkaline pH on the stimulation of glucose transport in rat skeletal muscle

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(Received 7 October 1992)

Key words: Epitrochlearis muscle; Hypoxia; Insulin; Muscle contraction; 3-*O*-Methylglucose

Alkaline pH has been reported to cause release of Ca^{2+} from skeletal muscle sarcoplasmic reticulum (SR). Elevation of sarcoplasmic Ca^{2+} concentration is thought to stimulate glucose transport in skeletal muscle. In this context, we examined the effect of alkaline pH (extracellular pH of 8.6) on 3-*O*-methylglucose transport in skeletal muscle. Incubation of rat epitrochlearis muscles at pH 8.6 for 45 min resulted in an approx. 3-fold increase in glucose transport activity, which was not affected by reducing Ca^{2+} concentration in the incubation medium and essentially completely blocked by 25 μM dantrolene, an inhibitor of SR Ca^{2+} release. In addition to stimulating glucose transport by itself, alkaline pH may partially inhibit the stimulation of sugar transport by insulin hypoxia and contractions, as the combined effect of alkaline pH and the maximal effect of insulin, contractions, or hypoxia on glucose transport are not different from the maximal effects of insulin, hypoxia, or contractions alone. The maximal effects of insulin and contractions, and of insulin and hypoxia, on glucose transport are normally additive in muscle. Alkaline pH completely prevented this additivity. In summary, our results show that alkaline pH stimulates glucose transport activity in skeletal muscle and provide evidence suggesting that this effect is mediated by Ca^{2+}. They further show that alkaline pH blocks the additivity of the maximal effects of insulin and contractions or hypoxia suggesting that alkaline pH may partially inhibit the stimulation of glucose transport by insulin, contraction and hypoxia.

Introduction

In skeletal muscle, glucose transport can be activated by insulin, contractile activity, and hypoxia. The maximal effects on glucose transport activity of insulin and contractions [1–4], and of insulin and hypoxia [5] are additive, while the maximal effects of contractions and hypoxia are not additive [5]. These findings have been taken as evidence that contractions and hypoxia stimulate glucose transport by the same pathway, and that insulin and contractile activity/hypoxia stimulate glucose transport by different pathways [1,2,5].

Evidence has accumulated suggesting that an increase in cytoplasmic Ca^{2+} concentration stimulates glucose transport in skeletal muscle and that this is the mechanism by which muscle contractions and hypoxia induce an increase in glucose transport activity [5–14]. Alkaline pH triggers Ca^{2+} release from skeletal muscle sarcoplasmic reticulum [15], and stimulates glucose transport in adipocytes and some cell lines [16–18]. However, we have not been able to find any information regarding the effect of alkaline pH on glucose transport in muscle. In this context, the present study was undertaken to determine whether or not alkaline pH stimulates glucose transport in skeletal muscle.

Our results show that alkaline pH does increase glucose transport activity in skeletal muscle and suggest that this effect is mediated by an increase in cytoplasmic Ca^{2+}.

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Methods

Animals and muscle preparation

Male Wistar rats weighing 100–120 g were obtained from SASCO (Omaha, NE) and fed Purina Chow and water. After an overnight fast, rats were anesthetized with 5 mg/100 g body wt. of pentobarbital sodium injected intraperitoneally, and the epitrochlearis muscles [19] were dissected out.

Muscle incubations

The muscles were incubated in 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) [20] containing 8 mM glucose, 32 mM mannitol, 0.1% bovine serum albumin and the additions indicated for each experi-

ment, in 25 ml Erlenmeyer flasks in a shaking Dubnoff incubator at 35°C. The gas phase in the flasks under our control condition was 95% O_2-5% CO_2. Alkalinization of the medium was achieved by reducing the proportion of CO_2 in the gas mixture. Unless otherwise stated, the term 'alkaline pH' represents an incubation medium pH of 8.6 in this paper. The pH of the medium was determined with a pH electrode. Like the effects of hypoxia and contractions, the effect of alkaline pH on glucose transport lasts sufficiently long to make it possible to measure 10 min after removal of the stimulus, i.e., incubation at physiological pH.

For studies of the effect of hypoxia, muscles were incubated in medium pre-gassed with 95% N_2-5% CO_2 at 35°C for either 20 min (submaximal effect) or 60 min (maximal effect) in flasks with a gas phase of 95% N_2-5% CO_2. For studies of the effect of hypoxia plus alkaline pH the gas mixture was 99.8% N_2-0.2% CO_2.

Muscle contractions

For studies of the effect of contractile activity, muscles were electrically stimulated to contract in vitro as previously described [7,21]. Ten tetanic contractions were elicited by stimulating for 10 s at 100 Hz once per min for 10 min.

Measurement of 3-O-methylglucose (3-MG) transport into muscle

After the initial incubation period, the muscles were rinsed, to remove glucose, by shaking for 10 min at 29°C in 2 ml of KHB containing 40 mM mannitol. If insulin or vanadate was present in the initial incubation they were also included at the same concentration in the rinse medium. Following the rinse step, glucose transport activity was measured using the nonmetabolizable glucose analog 3-*O*-methylglucose as described previously [22]. The muscles were transferred to flasks with 1.5 ml KHB containing 8 mM 3-*O*-[^{3}H]methylglucose (300 μCi/mmol), 32 mM [^{14}C]mannitol (9.4 μCi/mmol) and the same concentrations of insulin or vanadate as during the preceding incubations. The flasks were incubated in a shaking incubator for 10 min at 29°C. The gas phase in the flasks during the rinse step and the measurement of 3-MG transport was 95% O_2-5% CO_2 and the KHB was pre-gassed with 95% O_2-5% CO_2. This was made possible by the finding that the effects of alkaline pH and hypoxia [5], like that of contractions [7], on glucose transport persists for sufficiently long to measure the effect after cessation of the stimulus. The muscles were processed, and the extracellular space and intracellular 3-MG concentration were determined, as previously described [22]. 3-MG transport is expressed as μmol of 3-MG accumulation per ml of intracellular water in 10 min.

Measurement of intracellular pH

The distribution of 5,5-[^{14}C]dimethyloxazolidine-2,4-dione ([^{14}C]DMO) between the intracellular and extracellular spaces was used to provide a measure of internal pH [23,24]. Medium CO_2 tension was adjusted to yield the desired external pH (95% O_2-5% CO_2 for pH 7.4; 99.8% O_2-0.2% CO_2 for pH 8.6). A value of 6.13 was used for the pK of DMO [24]. Muscles were incubated for 30 min in 1.5 ml of KHB containing 3 μCi [^{14}C]DMO (1 mM) and 1.5 μCi [^{3}H]mannitol (32 mM).

Determination of glycogen, ATP and creatine phosphate concentrations

Muscles were clamp frozen with tongs cooled in liquid N_2. Weighed portions of frozen muscle were homogenized in $HClO_4$ [25]. An aliquot of homogenate was used for measurement of glycogen concentration by the amyloglucosidase method [26]. The remainder of the homogenate was centrifuged at 3000 × g for 15 min at 4°C. The supernatant was neutralized and used for measurement of ATP [27] and creatine phosphate [28].

Statistics

Values are expressed as means ± S.E. Differences between mean values were assessed using Student's t-test. For multiple comparisons, significance was determined by analysis of variance (ANOVA). Significant differences between means were located using the Newman-Keul multiple comparison test.

Results

Effect of alkaline pH on glucose transport activity

As shown in Fig. 1, incubation of epitrochlearis muscles for 45 min at pH 8.6 resulted in an approx. 3-fold increase in the rate of 3-MG transport, while pH 9.1 caused an approx. 4-fold increase. Fig. 2 shows the

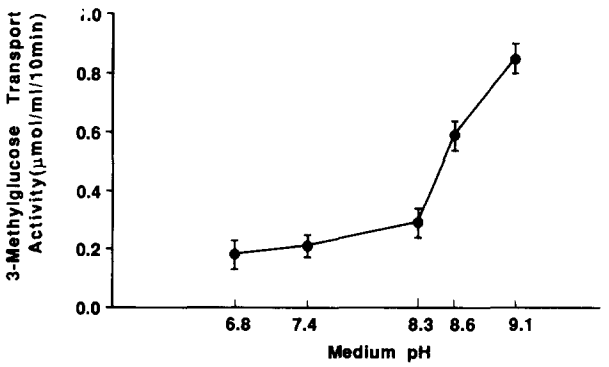

Fig. 1. Effect of altering the pH in the incubation medium on the rate of 3-methylglucose transport in epitrochlearis muscle. Muscles were incubated for 45 min in media maintained at the indicated pH, after which glucose transport activity was measured as described in Methods. Values are means ± S.E. for 10–11 muscles per point.

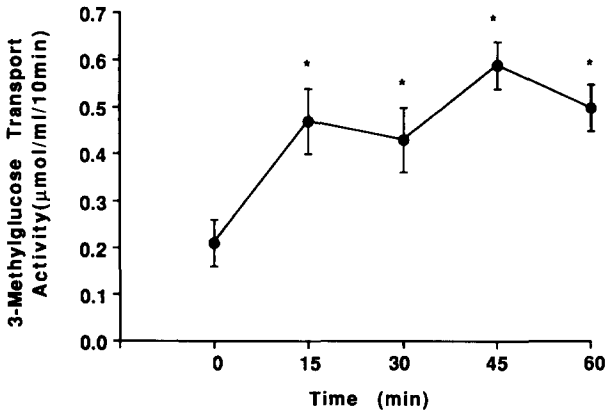

Fig. 2. Time course of 3-methylglucose transport activation by alkaline pH (8.6) in epitrochlearis muscle. After dissection, glucose transport activity was immediately measured in one group of muscles (time zero). Other muscles were incubated in medium maintained at pH 8.6 for the indicated times, after which glucose transport activity was measured as described in Methods. Values are means ± S.E. for 9–11 muscles per point. * Significant difference ($P < 0.05$) from time zero.

time course of the increase in glucose transport activity in muscles incubated at pH 8.6. The increase in transport activity occurs fairly rapidly, such that 3-MG transport was 2.2-fold higher after 15 min. The maximal stimulation of 3-MG transport was seen after 45 min. The increase in glucose transport activity induced by alkaline pH lasts sufficiently long to make it possible to measure 3-MG transport at a physiological pH, 10 min after removal from the alkaline pH medium. In all subsequent experiments involving the effects of alkaline pH, muscles were incubated at pH 8.6 for 45 min.

Effects of alkaline pH on muscle glycogen, ~P and pH_i

As shown in Table I, incubation of muscles for 45 min at pH 8.6 caused a small, but statistically significant decrease in glycogen concentration, but did not result in depletion of high energy phosphates (i.e., ATP + creatine phosphate). Estimation of intracellular pH from the distribution of the weak acid DMO between the intracellular space and the incubation medium, gave a value of 8.2 in muscles incubated for 45 min in medium with a pH of 8.6. Addition of 25 μM dantrolene to the alkaline pH medium did not interfere with the rise in intracellular pH.

Role of Ca^{2+} in the stimulation of glucose transport activity by alkaline pH

There is evidence suggesting that an increase in cytoplasmic Ca^{2+} concentration stimulates glucose transport in muscle and that the initiating event in the stimulation of glucose transport activity in skeletal muscle by contractile activity and hypoxia is an increase in cytoplasmic Ca^{2+} [5–14]. Experiments were therefore conducted to obtain information regarding whether or not an increase in cytoplasmic Ca^{2+} is involved in the stimulation of glucose transport activity by alkaline pH.

Incubation of muscles in low Ca^{2+} (medium made from KHB containing only 0.1 mM Ca^{2+}) did not alter the rate of 3-MG transport in the basal state or after incubation at alkaline pH (Table II). It was previously found that enhancement of 3-MG transport by insulin in epitrochlearis muscles is not impaired by low Ca^{2+} in the incubation medium [7]. Treatment with dantrolene, an agent that inhibits release of Ca^{2+} from the sarcoplasmic reticulum in skeletal muscle [29], inhibited the stimulation of 3-MG transport by alkaline pH (Table II).

Interaction of alkaline pH with insulin and vanadate

The combined effect of alkaline pH and a submaximally effective insulin concentration, 50 μU/ml, on 3-MG transport was significantly greater than that of alkaline pH alone, but less than fully additive (Table III). The combined effect of alkaline pH and a maximally effective insulin stimulus, 20 mU/ml, on 3-MG transport was not significantly different from that of 20 mU/ml of insulin alone (Table III).

TABLE I

Effect of external alkaline pH on muscle glycogen, ATP, phosphocreatine and intracellular pH

Values are means ± S.E. ($n = 6$). Muscles were incubated for 45 min at either pH 7.4 or 8.6. All metabolite concentrations are expressed as μmol/g wt.

Medium pH	Glycogen	ATP	PCr	pH_i
pH 7.4	17.5 ± 1.1	6.0 ± 0.3	16.5 ± 1.4	7.30 ± 0.01
pH 8.6	14.3 ± 1.0 *	5.7 ± 0.4	19.4 ± 1.4	8.20 ± 0.02 *
pH 8.6 + dantrolene (25 μM)				8.14 ± 0.04 *

* $P < 0.05$, pH 8.6 vs. pH 7.4.

TABLE II

Effects of dantrolene and of low extracellular Ca^{2+} on stimulation of 3-methylglucose transport by alkaline pH

Values are means ± S.E. for the number of muscles given in parentheses. Low Ca^{2+} incubation medium refers to incubation medium made up to contain 0.1 mM Ca^{2+}. All other media contained 2.5 mM Ca^{2+}.

Treatment	3-Methylglucose transport (μmol/ml per 10 min)	
None	0.23 ± 0.06	(12)
Low Ca^{2+} incubation medium	0.23 ± 0.04	(6)
Alkaline pH	0.59 ± 0.08	(6)
Alkaline pH + low Ca^{2+} incubation medium	0.54 ± 0.03	(6)
Alkaline pH + dantrolene, 25 μM	0.31 ± 0.04 *	(11)

* $P < 0.05$ vs. alkaline pH.

Alkaline pH has been shown to interfere with the interaction of insulin with its receptor in the cell membrane. We next used vanadate, which mimics the action of insulin on glucose transport in skeletal muscle [30] but appears to act at a post-insulin receptor binding step [31]. As shown in Table III, the effects of vanadate and alkaline pH together were not significantly different from that of vanadate alone.

Interactions between alkaline pH and contractions or hypoxia

When muscles that had been exposed to alkaline pH were stimulated to contract using a maximally effective protocol, the increase in 3-MG transport was no greater than that induced by contractile activity alone, i.e., at pH 7.4 (Table IV). The tetanic stimuli, which were applied during the last 10 min of the 45 min incubation period, resulted in generation of only ~ 50–60% as much contractile force in the muscles incubated at pH 8.6 as in the paired muscles incubated at pH 7.4. We therefore also examined the effect of hypoxia, which appears to stimulate glucose transport by the same pathway as contractile activity [5].

The combined effect of alkaline pH and a maximally effective period of hypoxia, resulted in an increase in 3-MG transport that was not significantly different from that induced by the hypoxia alone (Table IV). The effect of alkaline pH combined with a submaximally effective period of hypoxia, 20 min, on 3-MG transport was significantly greater than that of either of these stimuli alone, but less than completely additive (Table IV).

Alkaline pH, insulin and contractions or hypoxia in combination

As in previous studies [1–5], the maximal increases in 3-MG transport induced by insulin and contractions, and by insulin and hypoxia, were completely additive (Table V). Alkaline pH completely prevented this additivity, so that muscles treated with insulin plus contractions or hypoxia in alkaline medium showed no greater increase in glucose transport activity than muscles treated with any one of these stimuli alone at pH 7.4 (Table V).

TABLE III

Interaction between the effects of alkaline pH and insulin or vanadate on 3-methylglucose transport in epitrochlearis muscle

Values are means ± S.E. for the number of muscles given in parentheses.

Treatment	3-Methylglucose transport (μmol/ml per 10 min)	
None	0.20 ± 0.04	(6)
Alkaline pH	0.57 ± 0.08	(6)
Insulin, 50 μU/ml	0.46 ± 0.06	(5)
Alkaline pH + insulin, 50 μU/ml	0.73 ± 0.08 *	(5)
Insulin, 20 mU/ml	1.40 ± 0.18	(6)
Alkaline pH + insulin, 20 mU/ml	1.31 ± 0.23	(5)
Vanadate, 10 mM	0.91 ± 0.20	(6)
Alkaline pH + vanadate, 10 mM	0.98 ± 0.11	(6)

* $P < 0.05$ vs. alkaline pH.

TABLE IV

Interaction between the effects of alkaline pH and contractions or hypoxia on 3-methylglucose transport

Values are means ± S.E. for the number of muscles shown in parentheses.

Treatment	3-Methylglucose transport (μmol/ml per 10 min)	
None	0.21 ± 0.04	(6)
Alkaline pH	0.67 ± 0.12	(6)
Contractions	1.14 ± 0.11	(6)
Alkaline pH + contractions	1.09 ± 0.15	(6)
Hypoxia, 20 min	0.62 ± 0.07	(9)
Alkaline pH + hypoxia, 20 min	0.82 ± 0.04 *	(9)
Hypoxia, 60 min	1.32 ± 0.19	(6)
Alkaline pH + hypoxia, 60 min	1.19 ± 0.18	(6)

* Alkaline pH + hypoxia, 20 min vs. alkaline pH or hypoxia, 20 min, $P < 0.05$.

Effect of methylamine on pHi and glucose transport

In order to achieve intracellular alkalinization by a method other than raising pH in extracellular medium, we incubated muscles in KHB (pH 7.4) containing 20 or 40 mM methylamine. Intracellular pH estimated by DMO increased 0.14 units after 45 min of incubation with 20 mM methylamine. A further rise in pHi (0.11 units) was noted after 45 min of incubation with 40 mM methylamine. Determination of 3-methylglucose transport activity revealed a small increase in response to 20 mM methylamine (from 0.17 ± 0.06 to 0.27 ± 0.07

TABLE V

Inhibition by alkaline pH of the additive effect of insulin and contractions or hypoxia on 3-methylglucose transport

Values are means ± S.E. for the number of muscles shown in parentheses.

Treatment	pH	3-Methylglucose transport (μmol/ml per 10 min)	
None	7.4	0.21 ± 0.04	(9)
	8.6	0.61 ± 0.05	(11)
Insulin, 20 mU/ml	7.4	1.01 ± 0.13	(5)
Contractions	7.4	1.14 ± 0.11	(6)
Hypoxia, 60 min	7.4	1.12 ± 0.09	(6)
Insulin + contractions	7.4	2.01 ± 0.20 *	(5)
	8.6	1.17 ± 0.11 +	(5)
Insulin + hypoxia	7.4	1.93 ± 0.11 *	(14)
	8.6	1.06 ± 0.10 +	(14)

* Significantly different from insulin, contractions, hypoxia, $P < 0.01$.
+ Significantly different from pH 7.4, $P < 0.01$.

μmol/ml per 10 min, $P > 0.05$). No further increase in transport activity was observed with 40 mM methylamine.

Discussion

The results of this study show that, as in several other cell types [16–18], alkaline pH results in stimulation of glucose transport activity in skeletal muscle. Available evidence suggests that glucose transport can be activated via two separate pathways in skeletal muscle. One of these is activated by insulin and insulin mimetic agents such as vanadate, while the other is activated by muscle contractions and hypoxia [1–5]. There is evidence that an increase in cytoplasmic Ca^{2+} concentration is involved in the stimulation of glucose transport by muscle contractions and hypoxia [5–11,13,14], and that increases in cytoplasmic Ca^{2+} concentration too low to cause contraction can cause an increase in glucose transport activity in skeletal muscle [12]. We therefore examined the possibility that Ca^{2+} is involved in the stimulation of glucose transport by alkaline pH. A 96% reduction in the Ca^{2+} concentration of the incubation medium had no effect on the stimulation of glucose transport by alkaline pH. Similarly, omission of Ca^{2+} from the medium has no significant effect on the stimulation of glucose transport activity in skeletal muscle by insulin [7]. However, dantrolene, which inhibits Ca^{2+} release from the sarcoplasmic reticulum [29] markedly inhibited the stimulation of glucose transport activity by alkaline pH. Taken together, these findings support the hypothesis that alkaline pH, like contractile activity, hypoxia, and phospholipase C and W-7 [5–14] stimulates glucose transport by releasing Ca^{2+} from the sarcoplasmic reticulum.

Available evidence suggests that glucose transport can be activated via two separate pathways in skeletal muscle. One of these is activated by insulin and insulin mimetic agents such as vanadate, while the other is activated by muscle contractions and hypoxia [1–5]. Evidence that different pathways are involved is provided by the finding that the maximal effects on glucose transport activity of insulin and muscle contractions [1–4] and of insulin and hypoxia [5] are additive, while the maximal effects of contractile activity and hypoxia are not [5]. The results from the present study demonstrate that alkaline pH partially inhibits both submaximal and maximal stimulation of glucose transport via the two pathways. This finding provides evidence that alkaline pH inhibits a step that is common to the two pathways for stimulating glucose transport in striated muscle. As a consequence of the inhibition, the maximal effects of insulin, contractions, or hypoxia in combination with alkaline pH were not significantly different from the maximal effects of each of these stimuli alone, i.e., at physiological pH. In addition to its partial inhibition of the stimulation of glucose transport by these agents, we found that alkaline pH has the interesting effect of completely blocking the additivity of the maximal effects of insulin and contractions, and of insulin and hypoxia, on glucose transport.

Studies on L_6H_9 skeletal muscle cells [32] and 3T3-LI fibroblasts [33] have shown that insulin, in addition to increasing glucose transport activity, also raises intracellular pH. The small increase in intracellular pH (0.11 units) by insulin can be dissociated from insulin's action on glucose transport, as prevention of alkalinization by a number of compounds, has no effect on insulin-stimulated glucose transport [32,33]. Similarly, incubation of muscles with 40 mM methylamine, which raised intracellular pH by 0.25 units, did not significantly stimulate glucose transport. These findings suggest that the threshold for activation of glucose transport by intracellular alkalinization is greater than 0.25 pH units.

Available evidence indicates that insulin as well as contractions cause a translocation of glucose transporters from an intracellular site into the plasma membrane in skeletal muscle [34–41]. The increase in glucose transport activity induced by insulin and contractions also appears to be associated with an increase in intrinsic activity of the transporter [38,42,43]. In this context, a possible explanation for the findings that alkaline pH (a) partially inhibits the stimulation of glucose transport by insulin, contractions and hypoxia, and (b) blocks the additivity of the effects of insulin and contractions or hypoxia, could be that alkaline pH prevents the increase in the intrinsic activity of the glucose transporters normally induced by these agents. The basis for the interpretation that alkaline pH may act by preventing induction of an increase in glucose transporter intrinsic activity, rather than by a direct inhibitory action on the glucose transporter, is that glucose transport was measured at physiological pH after the muscles were removed from alkaline pH and washed at pH 7.4 for 10 min. In other words, muscles were incubated at alkaline pH during the stimulation of glucose transport, not during its measurement.

The alternative possibility is that alkaline pH inhibits glucose transporter translocation from intracellular stores into the plasma membrane. Although alkaline pH inhibited the additivity of the effects of maximal insulin and contractile activity/hypoxia stimuli, the increase in glucose transport activity was still ~ 5-fold (Table V). Immuno-electronmicroscopy has shown that there are hardly any glucose transporters in the plasma membrane of skeletal muscle in the basal state [44], making it unlikely that the ~ 5-fold increase in glucose transport activity could have been due solely to an increase in intrinsic activity. Therefore, if alkaline pH acts by inhibiting glucose transporter translocation,

the inhibition would have to be partial. However, the finding that alkaline pH, by itself, stimulates glucose transport ~ 3-fold appears to argue against an inhibition of translocation. Clearly, clarification of this question will require further study after better methods have been developed for quantifying glucose transporter translocation in skeletal muscle.

In conclusion, the results of this study provide evidence that alkaline pH has two separate effects on the glucose transport process in skeletal muscle. One is to stimulate glucose transport activity, apparently via a Ca^{2+} dependent mechanism. The second is to partially inhibit the stimulation of glucose transport by both the insulin/vanadate and the contractile activity/hypoxia mediated pathways. The third is to block the additivity of the maximal effects of insulin and contractile activity/hypoxia on glucose transport activity.

Acknowledgements

We are grateful to Xian Jing Wang and Lori Nolte for excellent technical assistance. This research was supported by National Institute of Diabetes and Digestive and Kidney Diseases Research Grant DK-18986. E.J.H. was supported by National Institute on Aging Institutional Research Service Award AG-00078. E.A.G. was supported by National Institute on Aging Individual National Research Service Award AG-05404.

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