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Biochimica et Biophysica Acta 1510 (2001) 342–353

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Oxidative and drug-induced alterations in brush border membrane hemileaflet fluidity, functional consequences for glucose transport

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Received 3 August 2000; received in revised form 3 November 2000; accepted 3 November 2000

Abstract

Oxidation of biological membranes has been suggested as a major pathological process in a variety of disease states including intestinal ischemia and inflammatory bowel disease. Previous studies on the small intestinal brush border membrane have shown that part of the decrease in the activity of the Na^+-dependent glucose transporter (SGLT1) observed after oxidation could be secondary to the derangement in membrane fluidity that accompanied oxidative damage. The present study examined the relationship between oxidative-induced hemileaflet fluidity alterations and the resultant change in Na^+-dependent glucose transport activity. To address this issue, in vitro oxidation of guinea pig brush border membrane vesicles was induced by incubation of the vesicles with ferrous sulfate and ascorbate. We found that oxidation decreased the fluidity of both the outer and inner hemileaflets, the decrease being greater in the outer leaflet. Moreover, the preferential alteration in hemileaflet fluidity was accompanied by a decrease in glucose transport. However, when membrane perturbing agents such as hexanol and A_2C were used to restore membrane fluidity to levels comparable to controls, rates of glucose transport could not be interpreted in terms of variation of bulk membrane fluidity or variation in fluidity of any specific membrane leaflet. On the basis of these experiments, we propose that previous studies that reported coincidental alteration in membrane fluidity and glucose transport cannot be interpreted on the basis of bulk fluidity or hemileaflet fluidity.

Keywords: Oxidative stress; Peroxidation; Na^+-dependent glucose transport; SGLT1; Fluidity; Brush border membrane; 2-(2-Methoxyethoxy) ethyl 8-(*cis*-2-*n*-octyl-cyclopropyl) octanoate; Hexanol; 1,6-Diphenyl-1,3,5-hexatriene; [4-(Trimethyl-amino)phenyl]-6-phenyl-1,3,5-hexatriene

1. Introduction

The clinically significant malabsorption that accompanies inflammatory bowel disease may be promoted by deleterious oxidants released from inflammatory cells that accumulate within the intestinal interstitium during active disease [1]. Since the intestinal brush border membrane is of critical importance for the normal absorption of dietary nutrients, we have been interested in the effect of oxidation on rates of nutrient transport by this membrane. In this context, one of the mechanisms involved in oxidant-induced injury may represent either direct oxidative damage to the brush border membrane nutrient transporters or oxidation of the membrane lipids with a resultant alteration in membrane fluidity.

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0005-2736/01/$ – see front matter
PII: S0005-2736(00)00367-9

In a previous study, we demonstrated that in vitro oxidation of brush border membrane vesicles prompted a marked reduction in rates of glucose transport with a coincidental alteration in membrane fluidity [2]. The most striking finding in this study was that by returning membrane fluidity to values seen in control membranes we were able to recover a significant proportion of glucose transport activity. This implied that loss of transporter function was not solely a consequence of damage to the transport protein but was also dependent upon the lipid bilayer in which the transporter is embedded. Furthermore, these results were obtained by fluidizing the membrane with the fatty acid-like compound 2-(2-methoxyethoxy) ethyl 8-(*cis*-2-*n*-octyl-cyclopropyl) octanoate (A_2C), a membrane perturbing agent previously suggested to be preferentially active within the inner hemileaflet [3]. This forced us to entertain two hypotheses. Firstly, oxidative stress could primarily cause damage to the inner hemileaflet. Secondly, oxidative damage could be equivalent to each hemileaflet but the functional activity of the glucose transporter might be primarily dependent upon the fluidity of the inner hemileaflet. Furthermore, despite equivalent damage from the oxidative process, membrane fluidity might preferentially be disrupted in one hemileaflet. Either hypothesis or perhaps a combination of both could explain our observation and, therefore, we began a series of experiments to test each.

We first evaluated whether oxidative attack, induced from the luminal surface of the membrane, preferentially occurs in the inner hemileaflet and found that this was not the case. These data are reported elsewhere [4]. In the present study, we examined the effect of oxidation on the fluidity of each brush border membrane leaflet as well as the functional consequences of such alterations in terms of glucose transport. We tested the hypothesis that the functional activity of the glucose transporter depends upon the fluidity of one leaflet more than the other under both oxidative and normal conditions. This hypothesis has the advantage that it may explain some of the conflicting data in the literature concerning the role of membrane fluidity in modulating the Na^+-dependent glucose transporter activity. A variety of groups have examined this problem and there are data suggesting that transport rates either increase or decrease with increasing membrane fluidity [5,6]. In these studies bulk phase fluidity has been determined with no attention given to specific alterations in either hemileaflet. It is entirely possible that these superficially divergent results are consistent and what was overlooked was a common alteration in one hemileaflet that was obscured by different changes in the physical properties of the other. Membrane perturbing agents used in all these studies are now appreciated to have preferential effects on individual hemileaflets [7,8].

2. Materials and methods

2.1. Materials

All chemicals were obtained from either Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were of the highest grade available. D-[^{3}H]Glucose was obtained from Du Pont De Nemours (Wilmington, DE) and used as supplied. Fluorescent probes were purchased from Molecular Probes (Junction City, OR) and also used as supplied.

Statistical analysis was performed using the statistical software Systat (Evanston, IL). Differences between means were evaluated by analysis of variance with specific differences tested using a Tukey analysis as a post hoc test. A value of $P < 0.05$ was considered significant.

2.2. Preparation of brush border membrane vesicles

Brush border membrane vesicles were prepared from the small intestine using a magnesium precipitation method. Male albino guinea pigs, 275–300 g, were fasted overnight and sacrificed with an overdose of pentobarbital. The small bowel was rapidly removed and rinsed with ice-cold saline. Mucosal scrapings were homogenized with a Waring blender in 30 ml of 300 mM mannitol and 12 mM Tris–HCl and 120 ml distilled water. The homogenate was incubated with 1.5 ml of 1 M $MgCl_2$ for 20 min. The preparation was then centrifuged in a Sorvall SS-34 rotor at $7500 \times g$ for 15 min at 4°C. The supernatant was centrifuged at $20\,000 \times g$ for 30 min. The resulting pellet was resuspended in 50 ml of 300 mM

mannitol, 10 mM Tris–HEPES and 100 mM KCl (pH 7.5) and homogenized with a glass Teflon homogenizer for six strokes. The homogenate was incubated with 0.5 ml of 1 M $MgCl_2$ for 20 min prior to centrifugation at $7500 \times g$ for 15 min. The supernatant was then centrifuged at $32\,500 \times g$ for 45 min. The pellet was resuspended in 0.4 ml of 300 mM mannitol, 10 mM Tris–HEPES and 100 mM KCl (pH 7.5) with a syringe and 25 gauge needle.

All measurements were normalized to membrane protein content as determined by the method of Lowry et al. [9]. Sucrase activity in both the homogenate and microvillus membrane fractions was determined by the method of Dalquihst [10]. Final ratios of the sucrase specific activity in the isolated microvillus membrane fractions to the initial homogenate fractions varied from 8 to 14. Using the Mg^{2+} precipitation procedure, the vesicles are right-side out and sealed better than 90–95%, as previously shown by the failure to increase sucrase activity after detergent treatment [11].

2.3. Oxidation

Oxidation was induced by incubation of the vesicle preparations (1 mg protein/ml) with 200 μM $FeSO_4$ and 2 mM ascorbic acid. The reaction mixture was agitated at 60 oscillations/min throughout the incubation period at 37°C in a shaking water bath. The occurrence of peroxidation was assessed by the measure of the amount of thiobarbituric acid reactive substances formed during the reaction [12]. For fluorescence and transport measurements, the reaction was terminated by dilution of the sample with a large volume of ice-cold 300 mM mannitol, 10 mM Tris–HEPES and 100 mM KCl (pH 7.5) and centrifuged at $32\,500 \times g$ for 45 min at 4°C. The pellet was resuspended in 0.5 ml of the same buffer.

2.4. Trinitrobenzenesulfonic acid labelling

The determination of the fluidity for each membrane leaflet was derived from a method previously established by Dudeja et al. [3] using trinitrobenzene sulfonic acid (TNBS) as a fluorescence quenching agent. This quencher can be covalently linked to the outer membrane hemileaflet (non-penetrating conditions) [13]. Under these conditions, only the fluorescence emitted by fluorescent probes located in the outer leaflet is quenched. Thus, we can directly measure the probe fluorescence from the inner leaflet as well as from the total membrane and calculate the fluorescence from the outer hemileaflet.

Oxidized or control brush border membrane vesicles (0.5 mg/ml final volume) were treated with TNBS under either non-penetrating conditions (0.25 mM TNBS, 4°C, 30 mM NaCl, 120 mM $NaHCO_3$, pH 8.2 for 1 h) or penetrating conditions (0.25 mM TNBS, 37°C, 30 mM NaCl, 120 mM $NaHCO_3$, pH 8.2 for 1 h). The trinitrophenylation reaction was terminated by addition of a large volume of ice-cold 150 mM Tris–HCl (pH 7.0). The reaction mixture was then centrifuged at $30\,000 \times g$ for 45 min and the pellet was resuspended in 10 mM Tris–HEPES (pH 7.5) for further analyses.

2.5. Fluorescence spectroscopy and differential phase fluorometry

The brush border membrane physical properties were assessed by using the lipid-soluble fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and [4-(trimethyl-amino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH). Information on the fluorophore motions were obtained from time resolved anisotropy decay measurements. In this case, the steady-state anisotropy r_s follows the modified Perrin equation [14]:

$$r_s = r_\infty + (r_0 - r_\infty)/6R\tau \quad (1)$$

where r_0 is the intrinsic anisotropy of the probe, r_∞ is the limiting hindered anisotropy, R is the rotational rate and τ is the lifetime of the excited state of the probe. In this equation, r_s is resolved into a static (r_∞) component and a dynamic component. The latter contribution is related to the probe rotational rate (R), while r_∞ is related to the degree to which the probe rotations are restricted by the molecular packing of the membrane lipids [15]. In the present study, the term 'fluidity' is used as the reciprocal of membrane structural order [16]. An increase in r_∞ suggests an increase in the molecular packing or a decrease in the fluidity of the surrounding lipids.

Fluorescence studies were performed at 37°C with an SLM-4800C spectrofluorometer (SLM-Aminco, Urbana, IL). Stock solutions of 2 mM DPH and

2 mM TMA-DPH were prepared in tetrahydrofuran and dimethylsulfoxide respectively. Aqueous solutions of both DPH and TMA-DPH were prepared daily and stored protected from light until used. In a typical experiment, brush border membranes equivalent to 200 μg protein were incubated in 3 ml buffer containing either 53 nM DPH or 53 nM TMA-DPH. The steady-state anisotropy r_s was calculated using the equation:

$$r_s = \frac{(I_v/I_h)_v - (I_v/I_h)_h}{(I_v/I_h)_v + 2(I_v/I_h)_h} \quad (2)$$

where $(I_v/I_h)_v$ and $(I_v/I_h)_h$ represent the ratios of vertically to horizontally polarized emission light from samples excited with vertically and horizontally polarized light. Controls containing either the membrane or the fluorophore alone were concurrently examined to correct for light scattering and intrinsic fluorescence.

The time resolved decay of both DPH and TMA-DPH was examined indirectly by measuring the phase difference between the parallel and perpendicular components of the fluorescent emission when the sample was excited with polarized sinusoidally modulated light (phase differential fluorometry) at 30 MHz. An r_0 value of 0.390 was used for both DPH and TMA-DPH [17]. The phase difference value was determined to yield R and τ [14]. The lifetime of the excited state of the probe was determined using phase and modulation techniques at 6, 18 and 30 MHz [18] against 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in ethanol. In case of multiple lifetimes, heterogeneity determination was accomplished by non-linear least squares analysis which provides both multiple lifetimes and their fractional values.

2.6. Glucose transport

Brush border membrane vesicles were resuspended to a final concentration of 7–10 mg/ml in 300 mM mannitol, 10 mM Tris–HEPES and 100 mM KCl (pH 7.5). All experiments were performed under voltage clamped conditions by the addition of valinomycin. Vesicle uptake of D-glucose was assessed using a rapid filtration technique and a 5 s time course. Transport was initiated by rapidly mixing 10 μl of vesicles with 50 μl of a solution containing 100 mM mannitol, 10 mM Tris–HEPES, 100 mM NaCl, 100 mM KCl and 4 μM D-[^{3}H]glucose (pH 7.5). Transport was stopped by rapid dilution of the reaction mixture with 4 ml of ice-cold stop solution containing 100 mM mannitol, 10 mM Tris–HEPES, 100 mM NaCl and 100 mM KCl (pH 7.5). The final solution was rapidly filtered through a 0.45 μm filter (Millipore/Continental Water Systems, Bedford, MA), washed three times with 2 ml of stop solution and counted in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA). Data are expressed as pmol D-[^{3}H]glucose taken up per minute per mg protein. Concurrent with each experiment, the volume of the vesicles was measured by allowing vesicles to equilibrate over a 2 h period before filtration. The final size of the vesicles did not differ between groups. In certain experiments the effect of the fluidizing agents A_2C and hexanol were also analyzed.

3. Results

3.1. Location of trinitrophenyl groups in brush order membrane

In order to examine individual hemileaflet fluidity, we utilized the selective quenching of DPH fluorescence by TNBS covalently linked to the outer hemileaflet of the membrane. Before accepting data provided by these experiments, it is mandatory to ensure that TNBS did not penetrate into the vesicles and label the inner hemileaflet. To this end, we resolved the different lifetime components of DPH as described in Section 2. The results of these experiments are illustrated in Table 1. When brush border membranes not exposed to TNBS were examined, the fluorescent lifetime was best expressed by a single

Table 1
DPH fluorescence lifetime in brush border membrane

Membrane	Fluorescence lifetime (ns)		
	Untreated	TNBS-treated	
Control	9.61 ± 0.25	9.08 ± 0.64	4.04 ± 0.63
Peroxidized	8.52 ± 0.69	10.30 ± 0.41	3.95 ± 0.26

The molar fractions of DPH with each lifetime on the TNBS-treated membrane varied between 45 and 55% leaflet. Values represent mean ± S.E.M. (n = 4–5).

component of approximately 9 ns for both the control and peroxidized membrane. TNBS treatment using penetrating conditions produced a single major component but with a shorter lifetime of 4 ns (data not shown), implying that the majority of DPH in the membrane had been quenched. Under non-penetrating conditions, two lifetime components, 4 and 10 ns (Table 1), were observed representing the lifetimes of the unquenched DPH in the inner leaflet and the quenched DPH in the outer leaflet, respectively. The fractional proportion of each lifetime varied between 45 and 55% ($n=6$). Thus, under these conditions, we can be confident that TNBS has bound covalently only to phospholipids in the outer leaflet and, therefore, DPH fluorescence measured under these conditions arises from the inner leaflet.

3.2. Fluidity of the oxidized membranes

Oxidation of the membrane preparations was induced by addition of ferrous sulfate and ascorbate (200 μM/2 mM). Such oxidative conditions have been proposed to decrease the fluorescence lifetime of the probe DPH [19]. In the present study, this would have dramatic consequences towards reporting oxidative-induced alterations in membrane fluidity since an accurate measure of the probe fluorescence lifetime is essential to evaluate both R and r_∞. However, as reported in Table 1, the fluorescence lifetime values were not significantly different between control and oxidized membranes suggesting that oxidation under our conditions did not alter DPH fluorescent properties.

Having ascertained that DPH fluorescence lifetime was not altered under the present conditions, the inner and outer brush border membrane leaflet fluidity was examined. The values for r_∞ (panel A) and R (panel B) are shown in Fig. 1 for the probe DPH. In control membranes, r_∞ was found to be significantly higher in the inner leaflet compared to the outer (0.238 ± 0.001 versus 0.178 ± 0.004, $P < 0.001$, $n=4$). These results suggest that the inner brush border membrane leaflet is less fluid than the outer in agreement with previous reports [3,8]. In the same manner, the rotational rate of DPH was lower in the inner leaflet suggesting again that the motional freedom of the probe is less in this leaflet.

For the oxidized membranes, r_∞ increased in both leaflets while R significantly decreased in the outer leaflet only. Although the increase in r_∞ was apparent in both leaflets, the magnitude of the change was greatest in the outer hemileaflet suggesting a preferential decrease in membrane fluidity in the outer hemileaflet after oxidation. These results were consis-

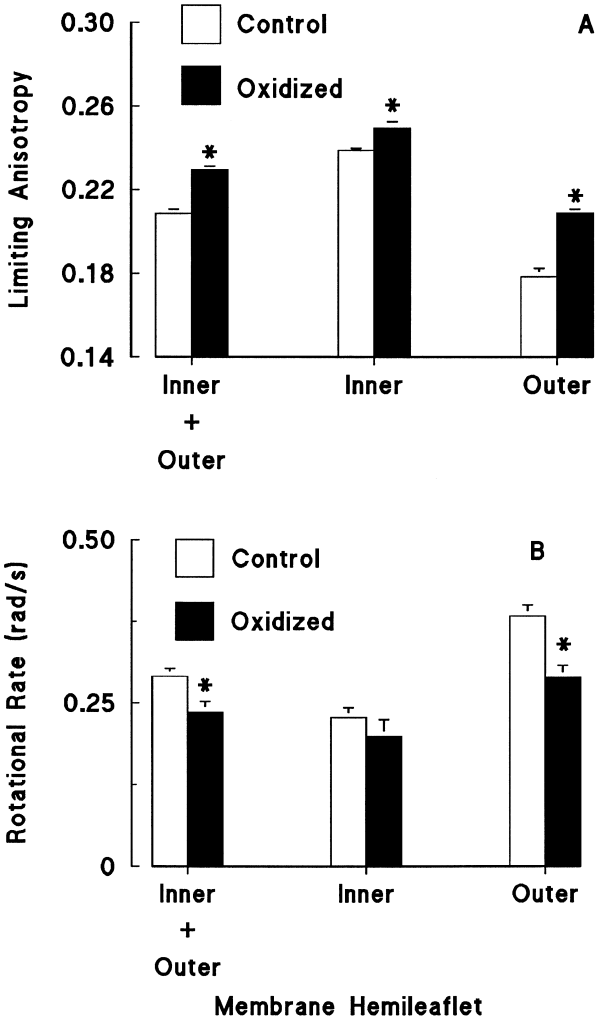

Fig. 1. Oxidation and individual hemileaflet physical properties. Brush border membrane vesicles were oxidized by addition of ascorbic acid (2 mM) and ferrous sulfate (200 μM) at 37°C. The samples were divided into two groups that were either untreated or treated with TNBS at 4°C (non-penetrating conditions). The limiting anisotropy r_∞ (A) and the rotational rate R (B) for the probe DPH were determined at 37°C by differential spectrofluorometry. r_∞ is related to the degree to which the fluorophore rotations are restricted by the molecular packing of the membrane lipids and R represents the rate of the probe rotation within the bilayer. An increase in r_∞ suggests a decrease in membrane fluidity. Values represent the mean ± S.E.M. ($n=4$–6). *$P < 0.05$ compared to control.

tent with our previous data showing that oxidative damage is greater in the outer hemileaflet of this membrane [4].

3.3. Bulk fluidity and glucose transport

We have already demonstrated that oxidation of the brush border membrane is accompanied by a reduction in rates of glucose transport that appeared in part to be secondary to the alteration in membrane fluidity [2]. Indeed, one-third of this reduction was reversible by fluidizing the vesicles and returning membrane fluidity to normal. To further evaluate the mechanisms underlying this observation we chose to utilize two different membrane fluidizers, the fatty acid-like molecule A_2C and the alcohol hexanol. These two lipophilic compounds, among others, have been reported to alter membrane fluidity as well as rates of glucose uptake into brush border membrane vesicles [3,5]. A_2C and hexanol were selected since neither of them altered the Na^+ gradient across the vesicle wall nor increased membrane permeability of water-soluble molecules [3,5]. Other drugs such as ethanol or benzyl alcohol have been demonstrated to have such confounding effects [5,20].

In order to compare the effects of both agents on rates of glucose transport, equieffective concentrations for each drug had to be found. We defined these by testing the effect of either drug on r_∞ for DPH, by selecting concentrations that returned the value of r_∞ of the oxidized membranes to that observed in control membranes. This is illustrated in Fig. 2A. The addition of 5 μM A_2C was effective in returning the bulk fluidity of oxidized membranes (solid bars) to the normal range (as defined by the opened bar labelled no addition with an upper and lower range shown by the dashed lines, representing the 95% confidence intervals of the control measurement). 5 mM hexanol was required to produce the same effect as illustrated in Fig. 2A. These concentrations of drugs effectively fluidized control membranes (open bars) as well, although the effect was more prominent for A_2C.

Fig. 2B illustrates the effects these manipulations had on rates of glucose transport in either control (open bars) or oxidized (solid bars) vesicles. Oxidized vesicles had lower rates of glucose uptake than respective controls. It is important to note that under the conditions utilized (labelled glucose without competing cold glucose) uptake rates are proportional to V_m/K_m. Since we have previously demonstrated that oxidation does not significantly alter the K_m [2], these uptake rates are proportional to the maximal glucose transport rate, V_m. Furthermore, these studies were

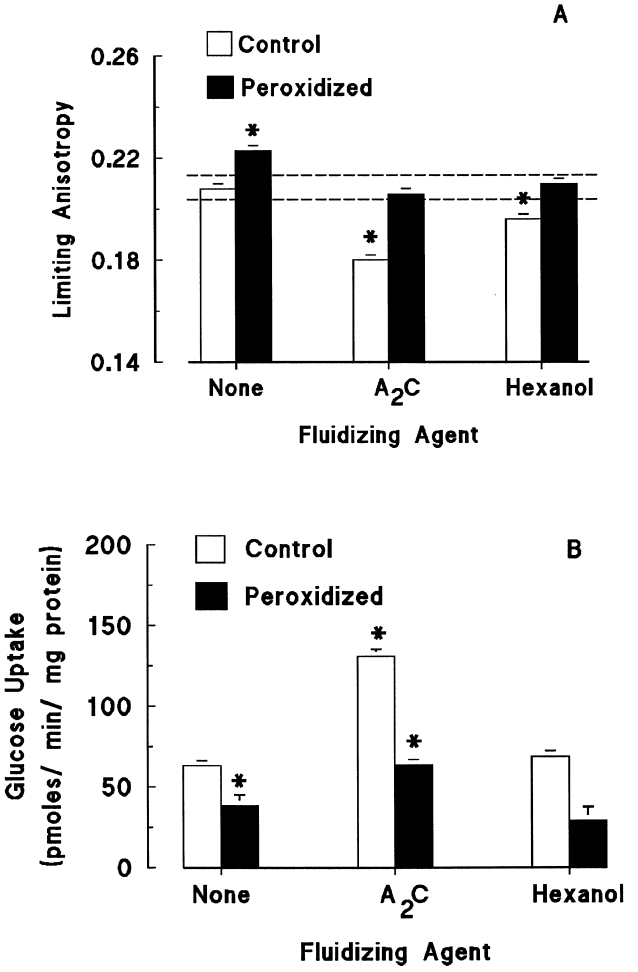

Fig. 2. Effect of the membrane perturbing agents A_2C and hexanol on the limiting anisotropy r_∞ of the probe DPH (A) and Na^+-dependent glucose uptake (B) in control and oxidized brush border membrane vesicles. (A) Dotted lines represent the 95% confidence intervals around r_∞ corresponding to the control conditions (no oxidation). When either 5 μM A_2C or 5 mM hexanol was added, r_∞ from oxidized membranes was not found significantly different from the control membranes. Values represent the mean ± S.E.M. ($n = 4$–6). *$P < 0.05$ compared to control. Under these conditions, rates of glucose transport were increased in the presence of A_2C while hexanol decreased them (B), an observation discussed more completely in the text. Values represent the mean ± S.E.M. ($n = 4$–6). *$P < 0.05$ compared to no addition of fluidizer.

performed under effective voltage clamped conditions with valinomycin and we have previously shown that Na^+ permeability is not affected by these manipulations [2]. This suggested that approximately 60% of the transporters were irreparably damaged during oxidation. It was also apparent that the remaining intact glucose transporters had their activity modulated by the membrane fluidity in a fashion very similar to that observed in control membranes. The addition of A_2C in the oxidized preparations significantly increased rates of transport to the level seen in controls. The same concentration of A_2C also significantly increased rates of glucose uptake in the control membranes.

While fluidization of the oxidized membranes with A_2C prompted an increase in rates of glucose uptake, fluidization with hexanol had no effect (Fig. 2B). A similar pattern was observed in control membranes since there was no significant difference in glucose uptake in the presence or the absence of hexanol. Therefore, it was evident that although rates of glucose transport in both oxidized and control vesicles could be modified by altering the membrane physical properties, these were not predictable with only data concerning the fluidity of the membrane as a single unit.

3.4. Hemileaflet fluidity and glucose transport

The fluidizer A_2C has been suggested to partition in the inner leaflet of plasma membranes [3] while hexanol may prefer the more fluid outer leaflet [7]. This would suggest that the increase in glucose transport observed in the presence of A_2C resulted from the preferential fluidization of the inner membrane leaflet. On the other hand, specific fluidization of the outer leaflet with hexanol would appear to have only minor effects since we observed no significant change in glucose uptake between control and hexanol-treated preparations (Fig. 2B). These observations raise the possibility that the functional activity of the glucose transporter may vary with the membrane leaflet fluidized. The experiments described below for native brush border membranes were performed to test this hypothesis.

To establish a relationship between hemileaflet fluidity and glucose transport, it was imperative to evaluate rates of glucose transport for graded concentrations of both A_2C and hexanol. Fig. 3 illustrates the data from these experiments. With increasing amounts of A_2C (panel A) rates of glucose uptake increased to a maximum (168.0 ± 10.0 versus 122.0 ± 4.0 pmol/min/mg for control, $n = 4$, $P < 0.05$) and then declined to rates observed in the absence of the drug. Changes in glucose transport in the presence of hexanol were also polyphasic (panel B). Glucose uptake was increased with 2.5 mM hexanol (63.16 ± 3.16 versus 98.06 ± 3.86 pmol/min/mg, $n = 4$, $P < 0.05$) while addition of either 10 or 15 mM hexanol significantly decreased glucose transport.

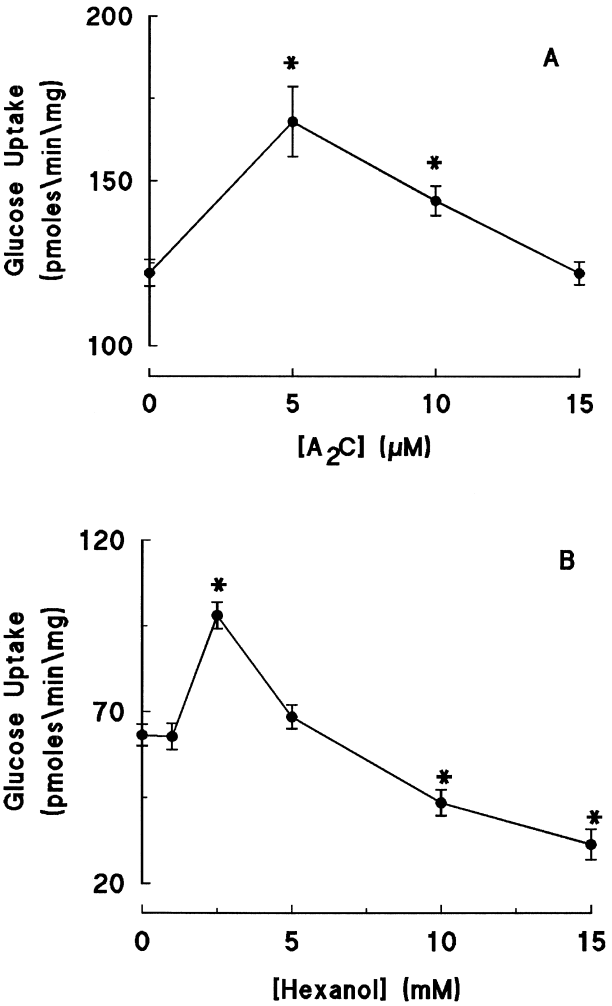

Fig. 3. Effect of A_2C (A) and hexanol (B) on Na^+-dependent glucose uptake in brush border membrane vesicles. Both A_2C and hexanol were able to increase rates of glucose uptake. Values represent the mean ± S.E.M. ($n = 4$–6). *$P < 0.05$ compared to control (no perturbing agent).

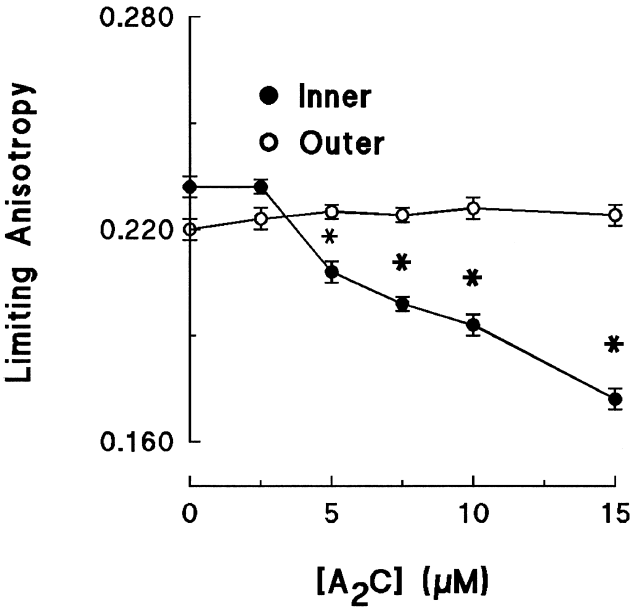

Fig. 4. Effect of A_2C on individual membrane fluidity. Membrane samples were divided into two groups that were either untreated or treated with TNBS at 4°C (non-penetrating conditions). The limiting anisotropy r_∞ for the probe DPH was determined at 37°C by differential spectrofluorometry for the inner hemileaflet (solid circles) and outer hemileaflet (open circles). Values represent the mean ± S.E.M. ($n=4$). *$P<0.05$ compared to control values for each leaflet.

The r_∞ values for DPH in the inner and outer membrane leaflets were evaluated under the same conditions and the results illustrated in Figs. 4 and 5, for A_2C and hexanol respectively. It was evident that A_2C specifically fluidized the inner membrane leaflet as shown by the concentration-dependent decrease in r_∞ in this leaflet only (Fig. 4). On the other hand, hexanol fluidized both the inner and outer membrane leaflets as suggested by the decrease in r_∞ in both leaflets (Fig. 5).

The apparent leaflet specificity of A_2C was quite puzzling since the fast transbilayer distribution of a fatty acid ester such as A_2C would preclude its preferential distribution in any particular leaflet. One possibility would be that the membrane perturbations induced by A_2C would vary with the region of the membrane probed and that some of these perturbations were overlooked by using DPH. Indeed, this probe lies close to the bilayer center, the information obtained from the probe fluorescence being restricted to this region of the membrane [21]. Because the fluorescent probe TMA-DPH senses the lipid–water interfacial region of the bilayer [22], a region different from that evaluated by DPH, we decided to examine the effect of A_2C using TMA-DPH.

To reliably interpret the results obtained with TMA-DPH, it was mandatory to verify the location of this probe in reference to each membrane leaflet. TMA-DPH is positively charged and it may partition preferentially in one membrane leaflet, e.g. the inner leaflet [7]. This was tested by utilizing the selective quenching of the probe fluorescence by trinitrophenyl groups as described. When the vesicles were treated with 0.25 mM TNBS at 4°C (non-penetrating conditions), the total fluorescence of the probe TMA-DPH was found to be one-half of the control preparation (53.0 ± 2.7%, $n=4$), while approximately 90% (89.3 ± 3.0%, $n=4$) of the probe fluorescence was quenched under penetrating conditions (37°C). Since quenching of TMA-DPH fluorescence occurs with high efficiency only over a short range (10 Å) insufficient to quench both sides of the membrane,

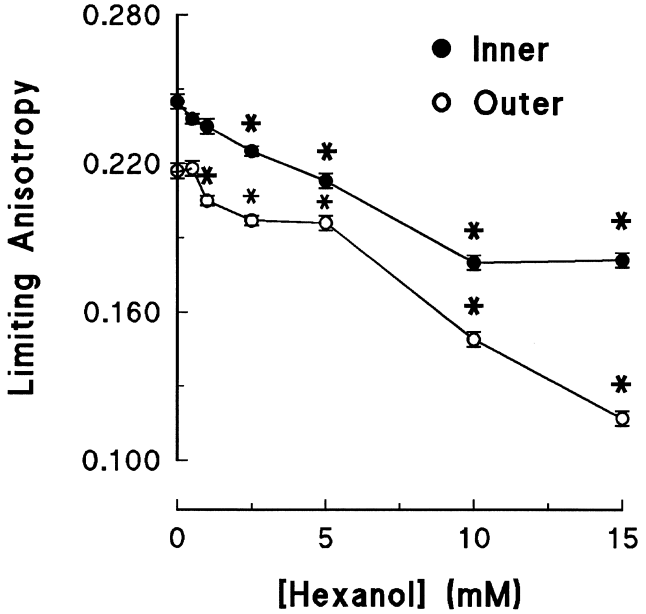

Fig. 5. Effect of hexanol on individual hemileaflet fluidity as determined with the probe DPH. Conditions were as described in Fig. 4. Values represent the mean ± S.E.M. ($n=4$). *$P<0.05$ compared to control values for each leaflet. Hexanol fluidized both leaflets.

these results were consistent with the interpretation that TMA-DPH partitions into the membrane in a symmetric manner. In other words, TMA-DPH did not localize in a particular membrane leaflet of our brush border membrane preparations.

The r_∞ values for TMA-DPH in the inner and outer membrane leaflets are depicted in Fig. 6 for increasing concentrations of A_2C. In the absence of fluidizer, the r_∞ values were higher than those obtained for DPH (Fig. 3) illustrating the high degree of packing observed in superficial regions compared to the membrane core, a concept known as fluidity gradient [23]. The addition of A_2C resulted in a statistically significant decrease of r_∞ in both leaflets for 2.5, 5 and 10 μM A_2C. These observations suggested that, in the presence of A_2C, the fluidity of the lipid–water interfacial region unlike the core region (as examined with DPH) was increased in both the inner and outer membrane leaflets. In the presence of 15 μM A_2C, the r_∞ values were not significantly different from those obtained for control conditions. The variations in r_∞ obtained for TMA-DPH did not exceed 5% of the control values compared to approximately 20% with DPH.

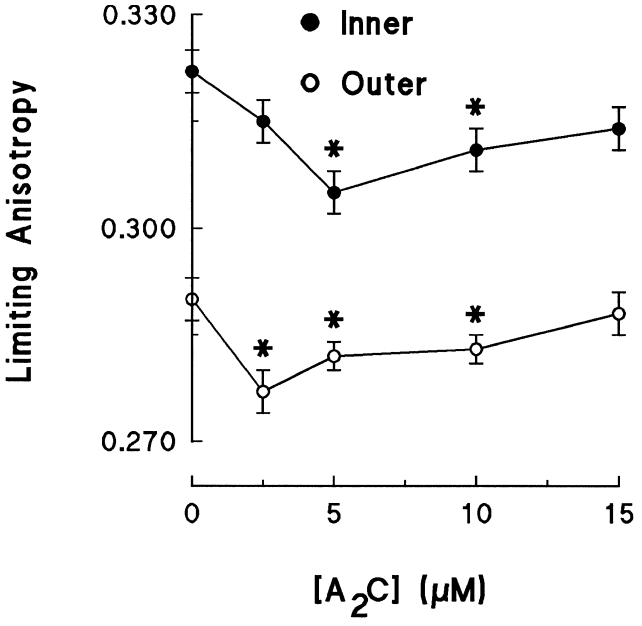

Fig. 6. Effect of A_2C on individual hemileaflet fluidity as determined with the probe TMA-DPH. Samples were treated as described in Fig. 4. The cationic charge of TMA-DPH ensures that this probe localizes to a more superficial region of the membrane bilayer compared to DPH. Values represent the mean ± S.E.M. ($n=4$). *$P<0.05$ compared to control values for each leaflet. A_2C did not specifically fluidize the inner hemileaflet when the membrane was probed with TMA-DPH.

The results illustrated in Fig. 5 for hexanol and Fig. 6 for A_2C clearly demonstrated that these compounds did not present any specificity for either hemileaflet. This left us with the difficult conclusion that the divergent results noted on glucose transport in both control and oxidized membranes cannot be interpreted in terms of variation of any specific leaflet.

4. Discussion

It has now been demonstrated that lipid peroxidation, initiated by several different mechanisms, reduces the fluidity of biological membranes. This observation represents an average alteration which does not take into account fluidity differences between the outer and inner membrane hemileaflets. Indeed, the two leaflets of mammalian plasma membranes, including the intestinal brush border membrane [24], are distinct in terms of lipid and protein composition, allowing each leaflet to have different fluidity [3]. Such asymmetry has been proposed as an important modulator of membrane functions including active and facilitated transport, receptor mobility, hormone–receptor interactions and membrane-bound enzyme activity [25,26]. Despite this, the effect of oxidation on hemileaflet fluidity as well as the functional consequences of such alteration have not been examined. In the present study, we report that oxidation of the intestinal brush border membrane is accompanied by a decrease in the fluidity of the two membrane leaflets, the decrease being greater in the outer. The selective alteration in leaflet fluidity was accompanied by a decrease in rates of glucose uptake into the same microvillus preparations.

Our results are in contrast to those of Schroeder et al. [27] who studied the effect of Fe(II)+H_2O_2 on fibroblast plasma membranes. Oxidation of these membranes prompted a decrease in the fluidity of the outer leaflet but not of the inner. However, in both this study and ours the outer leaflet was the most affected. This is not very surprising since in both cases the oxidizing agents were added directly to the membrane preparation, which exposed the

outer surface of the vesicles to direct oxidative damage. In a recent report, we also found that the outer hemileaflet of the small intestinal brush border membrane was more susceptible to oxidation than the inner [4]. These results were obtained by direct addition of the peroxyl radical generator (2,2′-azobis-(2-amidinopropane)-hydrochloric acid) on the membrane preparations. However, in this case the difference in leaflet susceptibility could not be ascribed to a variation in the oxidant concentration between the outer and inner membrane surfaces. It was rather related to a differential distribution of membrane antioxidants (such as vitamin E) and/or substrates for oxidation (such as unsaturated fatty acids) between the two leaflets.

Similarly, in addition to differences in oxidant availability, the oxidative-induced alteration in hemileaflet fluidity is likely to be secondary to several other molecular events. These include the loss, modification and relocation of membrane components important in determining membrane fluidity. Among these are unsaturated fatty acids and cholesterol, targets for oxidative attack and known to be asymmetrically distributed between hemileaflets [28]. Also important in the determination of hemileaflet fluidity is the asymmetric distribution of phospholipids. Their movements between hemileaflets (flip-flop) is increased in certain models of membrane oxidation and could account for our observations [29].

A major goal of the current experiments was to obtain a better understanding of the relationship between membrane fluidity and glucose transport under both oxidative and control conditions. Concomitant with the alteration in hemileaflet fluidity, the activity of the Na^+-dependent glucose transporter was drastically reduced following oxidation (Fig. 2). We have previously shown that neither an alteration of the Na^+-gradient across the vesicles nor a change in the passive permeability of the vesicles could account for the alteration in glucose transport. Furthermore, we also reported that a proportion of the lost glucose transport could be recovered by returning bulk membrane fluidity to the normal range [2]. To further investigate this observation, we elected to study the effect of two previously recognized fluidizers, A_2C and hexanol. The results of those experiments were illuminating. Concentrations of fluidizers that produced equivalent effects on bulk membrane fluidity were accompanied by divergent effects on rates of glucose transport: A_2C increased while hexanol decreased glucose transport (Fig. 2). This leads to the inescapable conclusion that simply determining bulk phase fluidity in the entire membrane cannot be used to predict the functional activity of this transporter.

An alternative explanation to these contradictory results would be that the transporter activity would depend upon the fluidity of one leaflet. This concept has been successfully tested for membrane proteins having their activity associated with either the outer or the inner side of plasma membranes [30]. A_2C and hexanol have been proposed to present different affinity for each leaflet of the intestinal brush border membrane [3,7,8]. Furthermore, opposite alteration in rates of glucose transport by A_2C and hexanol, as illustrated in Fig. 2, would suggest that independent alteration of membrane hemileaflet fluidity with either drug may result into differential alteration of the transporter activity. However, as clearly demonstrated in Figs. 4–6, neither A_2C nor hexanol exhibited any hemileaflet specificity at the concentrations used. Both A_2C and hexanol were able to increase glucose transport but at concentrations that differentially affected membrane fluidity when each individual leaflet were considered. Therefore, the hypothesis that the transporter activity may preferentially depend upon the fluidity of one leaflet was not supported by our results.

Over the past 20 years, a number of studies have explored the possibility that the brush border membrane fluidity is a primary determinant in modulating the Na^+-dependent glucose transporter [6,31]. However, despite intense research, the relationship between membrane fluidity and glucose transport is still a tenuous one. In light of the present experiments, we propose that rates of glucose transport cannot be interpreted in terms of simple response to bulk phase fluidity or even when individual membrane leaflet fluidity is considered.

Others have suggested that progress in understanding the relationship between membrane fluidity and membrane protein functions may require consideration of variations in bulk membrane fluidity in different regions of the membrane bilayer [23,32]. Interestingly, the specificity of A_2C for the inner hemileaflet was evident when the membrane was probed with

DPH but not with TMA-DPH. DPH localizes deep within the hydrophobic regions of membranes [21]. On the other hand, the cationic charge of TMA-DPH ensures that this probe partitions in more superficial regions, approximately to the level of the 8th to 10th carbon of phospholipid fatty acyl chains [22]. This is important as these observations suggest that the A_2C-induced perturbations vary when different depths of the bilayer (either deep or superficial) are assessed. Alcohols including hexanol also altered differentially lipid fluidity when either the superficial or the core region of the lipid bilayer was evaluated [33]. The relevance of these findings to the modulation of glucose transport by membrane fluidity is unclear. Further studies along these lines will therefore be of interest in our model.

Finally, can the present data confirm our original hypothesis that the activity of the glucose transporter depends upon the fluidity of one leaflet more than the other one during oxidative stress? The answer would appear to be no. Oxidation of the vesicular preparations was accompanied by a preferential reduction of the outer hemileaflet fluidity. However, differences in hemileaflet fluidity fail to entirely explain the restoration of glucose transport in the presence of A_2C. In fact, the comparison of the complex process of membrane oxidation with that of the addition of a lipophilic compound such as A_2C or hexanol might be an oversimplification. It is commonly accepted that the oxidative-induced decrease in membrane fluidity is the result of the decomposition of unsaturated fatty acids, a privileged target for oxidants and an important modulator of membrane fluidity. However, little or no attention has been given to the oxidation products and their possible interaction with membrane lipids and proteins. Malondialdehyde (MDA) is an important oxidation product responsible for some of the cytotoxic effects of lipid peroxidation [34]. Ohyashiki et al. [35] found that treatment of porcine intestinal brush border membrane with MDA resulted in a decrease in bulk fluidity. This was proposed to be due to the formation of intermolecular cross-links between membrane lipids. The oxidative-induced formation of protein aggregates has also been proposed to alter membrane fluidity [36]. Further studies are required to more fully identify the nature of these membrane-related disorders and to understand their involvement in diseases where oxidative damage is ongoing.

Acknowledgements

This work was funded by grants from the Medical Research Council of Canada and the Crohn's and Colitis Foundation of Canada. J.B.M. is an AHFMR scholar.

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