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Biochimica et Biophysica Acta 1372 (1998) 117–123

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Differential activation of system A and betaine/GABA transport in MDCK cell membranes by hypertonic stress

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Received 24 November 1997; revised 13 March 1998; accepted 17 March 1998

Abstract

Accumulation of osmolytes by renal cells is due in part to increased uptake via specific transporters. These include amino acid transport system A and the betaine/GABA transporter (BGT1). Transport changes have been characterized using intact cells which makes the intracellular mechanisms difficult to determine. In this study the hypertonic upregulation of system A and BGT1 was studied directly at the membrane level in Madin–Darby canine kidney (MDCK) cells. Both system A and BGT1 transport systems were detected in an isolated membrane fraction containing plasma membranes. System A transport was increased in membranes prepared from cells after 6 h hypertonic stress (449 mosmol/kg) but BGT1 activity was minimal and not different from isotonic controls. The increase in system A was blocked by inhibitors of RNA and protein synthesis. BGT1 transport was induced in membranes prepared after 24 h hypertonicity. At this time system A activity in the membrane fraction remained increased, unlike the downregulation observed in intact MDCK cells. We conclude that differential upregulation of system A and BGT1 by hypertonic stress is due to intrinsic changes in these transporters at the membrane level. In contrast, the downregulation of system A in intact cells when hypertonicity is prolonged for 24 h is likely due to the action of an intracellular repressor that is not present in the isolated membranes.

Keywords: α-(Methylamino)isobutyric acid; γ-Aminobutyric acid; Membrane transport; Osmotic stress; Actinomycin D; Cycloheximide

1. Introduction

Accumulation of inorganic ions is the rapid initial response to extracellular hypertonicity but high ionic strength has adverse effects on intracellular proteins [1]. When the hypertonicity is maintained the ions must be replaced by compatible organic solutes (osmolytes) that do not disturb protein function [2]. The complete adaptive response to a prolonged hypertonic challenge requires intracellular synthesis of specific osmolytes and expression of genes for specific osmolyte transporters [2]. Through these mechanisms cells can accumulate osmolytes in order to resist water exit and maintain a normal cell volume during sustained extracellular hypertonicity. The renal medulla is the only mammalian tissue that normally becomes hypertonic, as part of the urinary concentrating mechanism [2]. Betaine, sorbitol, inositol, taurine and glycerophosphorylcholine have been identified as major osmolytes in the renal medulla [3]. Madin–Darby canine kidney (MDCK) cells have been used widely to study osmoregulatory mechanisms in

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0005-2736/98/$19.00
PII S0005-2736(98)00051-0

renal epithelial cells. During hypertonic stress these cells accumulate betaine to concentrations that greatly exceed the extracellular betaine concentration due to increased activity of a Na^+/Cl^--dependent betaine transporter [2,3]. The transporter also accepts γ-aminobutyric acid (GABA) as a substrate and has been named the betaine/GABA transporter (BGT1) [4]. Upregulation of BGT1 was relatively slow, however, requiring 24 h of hypertonicity before a maximum was reached [2]. We [5] and others [6] previously showed that Na^+-dependent transport of amino acids via system A was upregulated in MDCK cells within 4–6 h of hypertonic treatment, well before there was any change in BGT1 activity [5]. Furthermore, this change in system A was accompanied by accumulation of alanine and other neutral amino acids [5,6], suggesting that system A substrates are used as osmolytes during the early phase of the adaptive response to prolonged hypertonicity. The transport changes have been characterized primarily in terms of cell uptake making the intracellular mechanisms difficult to determine. In the present study the adaptive responses of system A and BGT1 transporters in MDCK cells were studied directly at the membrane level. The purpose was to determine, in the absence of possible intracellular regulators, if intrinsic changes occurred in the membrane transport systems during hypertonic stress.

2. Methods

MDCK cells (CCL-34, American Type Culture Collection, Rockville, MD) were used between passages 10 and 25. They were plated in 35 mm plastic dishes (2.5×10^6 cells/dish) for cell uptake studies and in 75 cm^2 flasks (4×10^6 cells/flask) for membrane transport studies. The growth medium was a 1:1 mixture of DMEM and Ham's F-12K containing 10% bovine calf serum, 10 mM Hepes, 25 mM $NaHCO_3$ (pH 7.4), and penicillin G (100 U/ml), as in previous studies [5]. Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air and were used when confluent. The osmolality of the growth medium was 293 mosmol/kg, as determined by freezing point depression using an osmometer. Hypertonic growth medium was made by addition of either 200 mM sucrose or 100 mM NaCl to normal growth medium to achieve a final osmolality of 449 mosmol/kg. We showed previously [5] that hypertonic activation of system A was independent of the specific solute used to increase medium osmolality.

Na^+-dependent uptake of radiolabelled solutes by MDCK cell monolayers was determined by the procedure used and described previously [5,7]. The transport activities of system A and BGT1 were determined as cell uptake of α-[^{14}C]methylaminoisobutyric acid (MeAIB) and [^{3}H]GABA, respectively. Final concentrations of MeAIB and GABA in the uptake solutions were 0.1 mM and 0.01 mM, respectively [5,7]. Control cells maintained in isotonic growth medium were processed in parallel with the cells incubated in hypertonic medium, using isotonic or hypertonic uptake solution as appropriate [5,7]. All uptakes were determined at a 15 min incubation time which was shown previously to lie within the linear range for MDCK cells [5,6]. The data were corrected for surface binding and trapping of solute by subtraction of blank values.

Membrane fractions were prepared essentially as described for other renal epithelial cells [8] except that the cells were homogenized by nitrogen cavitation [9]. Briefly, confluent cell monolayers in four flasks (75 cm^2) were washed twice in Tris-buffered saline (pH 7.4) which was supplemented with 200 mM sucrose if the cells were previously incubated in hypertonic growth medium. The cells were collected from each flask by scraping in additional saline and were combined and recovered by low-speed centrifugation. The cell pellet was resuspended in 300 mM mannitol, 5 mM EGTA, 12 mM Tris–HCl (pH 7.1), containing 0.2 mM phenylmethylsulfonyl fluoride. The suspension was mixed with an equal volume of water and the cells were disrupted by nitrogen cavitation (Parr Instrument) after equilibration at 750 psi for 30 min. The homogenate was centrifuged at $3000 \times g$ for 10 min and the resultant supernatant was centrifuged at $48{,}000 \times g$ for 30 min to collect the membrane fraction. The membrane pellet was resuspended and dispersed in 300 mM mannitol, 12 mM Tris–HCl (pH 7.4) by 10 passes through a 26-gauge needle and was allowed to equilibrate for 2 h. The suspension was centrifuged again at $48{,}000 \times g$ for 30 min and the final pellet was resuspended in 0.1–0.2 ml mannitol–Tris buffer at a protein concentration of 5 mg/ml. All steps were performed at 4°C.

Membrane fractions were used immediately for solute uptake studies using the rapid filtration technique described in previous studies [9,10]. Uptake of solutes was determined at 25°C in medium containing 0.05 mg membrane protein, either NaCl or KCl (100 mM), mannitol (100 mM), 10 mM Tris (pH 7.4 with Hepes), and appropriate solute. Final solute concentration, including radioactive tracer, was either 0.05 mM ([^{14}C]MeAIB and [^{3}H]GABA) or 0.10 mM ($K_2H^{32}PO_4$).

Na^+/K^+-ATPase activity in cell fractions was assayed after detergent treatment to disrupt sealed vesicles, as described previously for MDCK cells [11]. Alkaline phosphatase [12] and protein [5,7] were determined as in earlier studies. All experiments were analyzed in triplicate and repeated at least three times. Control cells and hypertonic cells were always compared within the same experiment and results were analyzed with the Student *t*-test for paired comparisons. Differences were considered significant at $P < 0.05$.

3. Results

System A transport activity increased 2- to 3-fold within 4 h of switching MDCK cell monolayers to hypertonic medium. It reached a peak by 8 h and returned to the level in isotonic controls by 24 h (Fig. 1). In contrast, BGT1 activity showed no increase for up to 12 h after switching to hypertonic medium and remained similar to isotonic controls. Activation of BGT1 was not detected until after 18–24 h of hypertonic stress at which time there was a 3- to 4-fold increase (Fig. 1). Activation of BGT1 coincided with downregulation of system A. Transport activities in the isotonic controls did not change significantly throughout the 24 h period of the experiment. These findings confirm that the responses at the whole cell level are as expected, based on an earlier study in MDCK cells [5].

Na^+/K^+-ATPase activity was increased 4 ± 1 (mean $\pm$ S.D., $n = 3$) fold in the isolated membrane fraction compared to the starting homogenate, confirming the presence of membranes derived from the basolateral plasma membrane [12]. The activity of alkaline phosphatase, an apical plasma membrane marker enzyme in MDCK cells [13], was increased

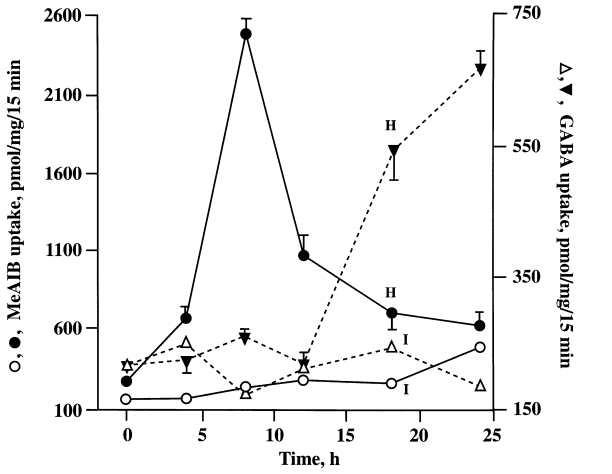

Fig. 1. A comparison of the time course of hypertonic activation of the transport activities of system A and BGT1 in MDCK monolayers. System A transport activity was determined as [^{14}C]MeAIB uptake (solid line) and BGT1 activity was determined as [^{3}H]GABA uptake (broken line), using Na^+ uptake medium. Uptakes were determined at various times after incubation in either hypertonic (H, 449 mosmol/kg, closed symbols) culture medium or isotonic (I, 293 mosmol/kg, open symbols) medium. Each point is the mean value of triplicate measurements. Bars indicating one standard deviation are shown only for hypertonic groups for clarity.

5 ± 3 (mean $\pm$ S.D., $n = 4$) fold in isolated membranes compared to homogenate. Both apical and basolateral plasma membranes are important sites of system A transport in polarized MDCK cells, based on flux measurements [6,12]. While BGT1 is exclusively basolateral under isotonic conditions, an apical activity is induced during hypertonic stress [14]. The membrane fraction is likely to contain a lower abundance of apical plasma membranes compared to basolateral plasma membranes, however, because morphometry has shown that the apical surface area of an MDCK cell in confluent culture is much less than the basolateral surface area [15].

Both BGT1 and system A transport activities were detected at the membrane level. Accumulation of [^{3}H]GABA by isolated membrane vesicles increased in a time-dependent manner and was stimulated by the presence of Na^+ in the extravesicular uptake medium (Fig. 2, left). The increase in uptake was linear for the first 60 s. The Na^+-dependency was greatly reduced by 120 min suggesting that uptake at this time was largely independent of the Na^+ gradi-

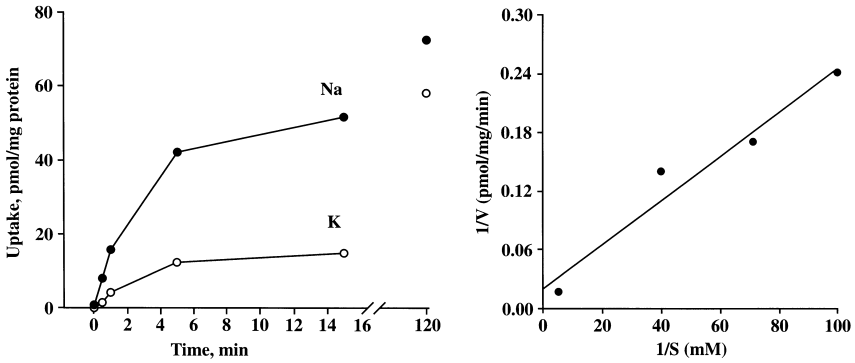

Fig. 2. BGT1 activity in a membrane fraction isolated from MDCK cells which were previously incubated in hypertonic culture medium for 24 h. Left panel: time course of [^{3}H]GABA uptake by isolated membranes in both Na^+ uptake medium (solid symbols) and when Na^+ was replaced by K^+ (open symbols). Right panel: double-reciprocal plot of initial phase (1 min uptake) of the specific Na^+-dependent component of [^{3}H]GABA uptake determined at GABA concentrations in the range 0.01–0.20 mM (correlation coefficient, $r = 0.98$). Each point is the mean value of triplicate measurements.

ent. The time course of [^{14}C]MeAIB uptake by the membrane fraction showed similar characteristics except that the presence of Na^+ produced a true 'overshoot' and there was complete equilibration by 90 min. At the peak of the overshoot the uptake of [^{14}C]MeAIB was twice the uptake at equilibrium (results not shown). The overall characteristics of system A transport are very similar to those reported previously [12] for an MDCK membrane fraction proposed to consist primarily of basolateral membrane vesicles. The data in Fig. 2 were obtained with membranes derived from hypertonically stressed cells. There was only minimal Na^+-dependent transport activity (both BGT1 and system A) in membranes from isotonic controls.

Membrane transport of [^{3}H]GABA was saturable and, based on 1 min uptakes, the Na^+-dependent component (total uptake in Na^+ medium minus uptake in K^+ medium) gave a straight line on a double-reciprocal plot (Fig. 2, right). The apparent K_m was 79 μM (mean of two experiments), very similar to the apparent K_m value of 81 μM (mean of two experiments) determined for Na^+-dependent [^{3}H]GABA uptake by intact cells after 24 h of hypertonic activation. Apparent V_{max} values were 32 and 15 pmol mg^{-1} min^{-1} for membranes and intact cells, respectively.

Isolated membrane fractions were used to determine if the hypertonic activation of system A and BGT1 transport activity in intact cells (Fig. 1) was due to an intrinsic change in the membrane-bound transporters. When membranes were prepared from MDCK cells after 6 h of hypertonic stress there was increased uptake of [^{14}C]MeAIB compared to isotonic controls, based on uptake measurements at 1 min (Fig. 3). The increased uptake was due entirely to a 4-fold activation of the Na^+-dependent component of [^{14}C]MeAIB uptake. There was no significant Na^+-dependent GABA uptake by membranes from isotonic control cells and no activation by 6 h of hyper-

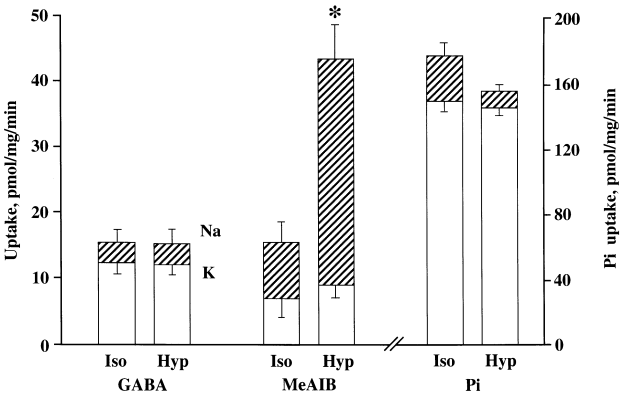

Fig. 3. Transport activity in membrane fractions isolated from MDCK cells after a 6 h incubation in either hypertonic (Hyp, 449 mosmol/kg) culture medium or isotonic (Iso, 293 mosmol/kg) medium. Uptakes were determined at 1 min both in the presence of Na^+ and when Na^+ was replaced by K^+ in the uptake medium. Shaded part of the columns represents the specific Na^+-dependent transport component. Data are mean ± S.D. of three different membrane preparations. * Significantly different ($P < 0.01$) compared to isotonic controls.

tonic stress. The membrane fraction also showed a small Na^+-dependent component of P_i uptake which was not activated after hypertonic stress (Fig. 3), suggesting that activation of system A was not due simply to changes in membrane permeability to Na^+. Total MeAIB uptake in Na^+ medium at the equilibrium point (90 min) was 58.6 ± 15.3 (isotonic) and 59.5 ± 18.6 (hypertonic) pmol mg^{-1} protein (mean $\pm$ S.D., $n = 3$). These values are not significantly different and suggest indirectly that the differences in MeAIB uptake at 1 min are not due to differences in intravesicular volume. In summary, the activation of system A with no change in BGT1 in an isolated membrane fraction closely reflects the transport changes observed in intact cells after 6 h of hypertonic stress (Fig. 1).

The presence of either actinomycin D (10 μM) or cycloheximide (20 μM) completely blocked the normal upregulation of system A in response to 5 h of hypertonic stress (Fig. 4). The treatment period was decreased in these experiments in order to avoid the cell rounding and detachment that were commonly observed during longer periods of hypertonicity in the presence of one of these inhibitors.

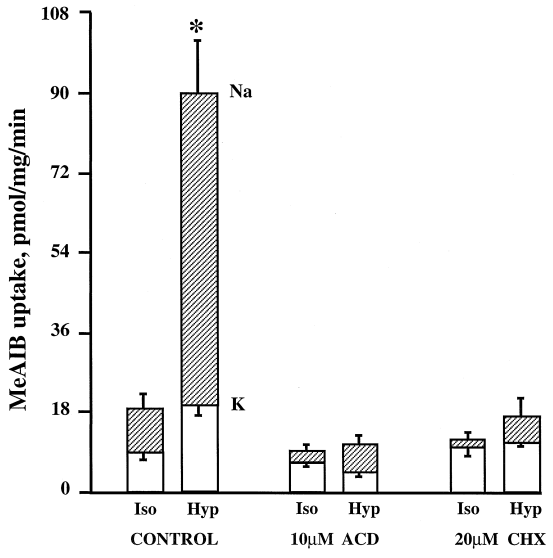

Fig. 4. System A transport activity in membrane fractions isolated from MDCK cells after a 5 h incubation in either hypertonic or isotonic culture medium. Where indicated, the Iso and Hyp media contained actinomycin D (ACD) or cycloheximide (CHX) at final concentrations of 10 and 20 μM, respectively. MeAIB uptake was determined at 1 min, during the initial linear phase of uptake. Other details as in Fig. 3. Data are mean + S.D. from three different membrane preparations. * Significantly different ($P < 0.02$) compared to isotonic control group.

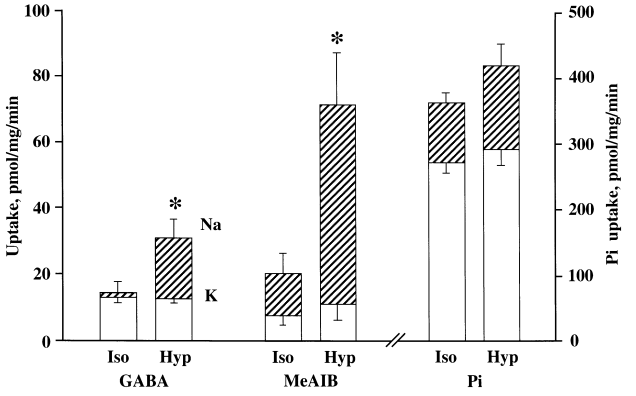

Fig. 5. Transport activity in membrane fractions isolated from MDCK cells after a 24 h incubation in either hypertonic culture medium or isotonic medium. Other details as in Fig. 3. Data are mean $\pm$ S.D. of four different membrane preparations. * Significantly different ($P < 0.05$) compared to isotonic controls.

Membrane fractions that were prepared from cells after 24 h of hypertonic stress exhibited increased uptake of [^{3}H]GABA. The increased uptake was accounted for completely by activation of a Na^+-dependent transport process which was not present in membranes from isotonic controls (Fig. 5). The membranes from hypertonic cells also showed increased uptake of [^{14}C]MeAIB that was due, as before (Fig. 3), to a 4–5 fold activation of the Na^+-dependent transport component. In contrast, there was no change in Na^+-dependent P_i transport after hypertonic stress (Fig. 5) indicating that the activation of GABA and MeAIB transport was not part of a widespread nonspecific adaptation of Na^+-dependent transporters. Neither GABA nor MeAIB uptake at the equilibrium point was significantly different between isotonic and hypertonic groups. For example, values for GABA uptake at 120 min (in Na^+ medium) were 64.9 ± 10.4 (isotonic) and 66.4 ± 2.1 (hypertonic) pmol mg^{-1} protein (mean $\pm$ S.D., $n = 4$). The increased GABA uptake at the membrane level after 24 h hypertonic stress reflects the activation of uptake in whole cells at this time (Fig. 1). In contrast to the downregulation of system A transport in intact cells after 24 h of hypertonic stress (Fig. 1), the Na^+-dependent MeAIB transport system in isolated membrane vesicles remained activated (Fig. 5).

It was not possible to determine if hypertonic activation of GABA uptake was blocked by actinomycin D or cycloheximide because the cells did not tolerate prolonged (>5 h) hypertonic stress in the presence of either of these inhibitors. The cells became rounded up and detached from the substrate.

4. Discussion

The observation that hypertonic activation of system A and BGT1 activity can be detected in an isolated membrane fraction indicates that the changes in whole cell uptake (Fig. 1) are likely mediated by specific changes in the properties of these membrane transport proteins. The activation at the membrane level involves upregulation of the Na^+-dependent transport component but it cannot be characterized in terms of V_{max} or K_m changes because the very low level of Na^+-dependent transport in the isotonic controls (Figs. 3 and 4) does not lend itself to kinetic studies. Thus it cannot be determined if upregulation is due to an increase in transporter efficiency or number. If the upregulation is due to interactions between the membrane transporter and a specific intracellular activator (with no change in overall transporter number), as has been proposed for system A [16,17], the interaction must result in covalent modification of the membrane transporter so that the upregulation persists in isolated membranes. An alternative possibility is that hypertonic upregulation of system A and BGT1 is due to an increase in transport number resulting from increased de novo synthesis of these specific proteins. This is supported by the observation that hypertonic upregulation of system A at both the membrane level (Fig. 4) and in intact MDCK cells [5] is blocked by inhibitors of RNA and protein synthesis. More extensive studies on BGT1 activity in MDCK cells have shown that hypertonic upregulation is associated with increased abundance of BGT1 specific mRNA due, in part, to increased gene transcription [18]. This is consistent with discovery of an osmotic response element that controls induction of BGT1 transcription by hypertonic stress [4].

While upregulation of system A and BGT1 is likely due to an increase in transporter number, the downregulation may involve a different mechanism at least for system A. The activity of this transporter in the membrane fraction remained upregulated after 24 h hypertonicity (Fig. 5) but system A transport in intact cells had decreased to control levels by this time (Fig. 1). This suggests, indirectly, that system A in whole cells may be repressed by an intracellular factor(s) produced during prolonged hypertonic stress. This mechanism of repression would not be detected in isolated membranes because the cytosolic components are washed away during membrane preparation. Another possibility for the apparent discrepancy is that it may be the result of using a heterogeneous membrane preparation that, although enriched in apical and basolateral plasma membranes, also contains membranes derived from intracellular structures. If the downregulation in intact cells is due to endocytic withdrawal of system A transporters, this shift from the plasma membrane pool to the intracellular membrane pool would not change total system A activity determined in a mixed membrane preparation containing both pools. Although there is an intracellular pool of system A transporters in both liver [19] and kidney [20] cells, this explanation seems unlikely because plasma membrane transporters that are endocytosed by kidney cells in response to a specific signal appear to be rapidly degraded, as demonstrated for the Na^+/phosphate cotransporter that is internalized in response to the action of parathyroid hormone [21].

In summary, upregulation of system A and BGT1 transport activity in MDCK cells during hypertonic stress is mediated by specific upregulation of these transporters at the membrane level. As in intact cells, upregulation of system A in isolated membranes required de novo synthesis of RNA and proteins and preceded the upregulation of BGT1 transport. However, the downregulation of system A after 24 h hypertonicity was not detected in isolated membranes suggesting indirectly that an intracellular repressor was required.

Acknowledgements

Part of this work was published previously in abstract form in *J. Am. Soc. Nephrol.* 8 (1997) 53A. I thank Dr. Jie-Guang Chen, Department of Pharmacology, Yale University, for helpful discussions, and

Dr. James McAteer, Department of Anatomy, Indiana University, for providing MDCK cells. Rose Hinesley and Karin Colmenares provided excellent technical assistance. Supported in part by a grant from the National Kidney Foundation of Indiana.

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