Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury

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Submitted 5 December 2006; accepted in final form 6 February 2007

Means CK, Xiao C-Y, Li Z, Zhang T, Omens JH, Ishii I, Chun J, Brown JH. Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 292: H2944–H2951, 2007. First published February 9, 2007; doi:10.1152/ajpheart.01331.2006.—Sphingosine 1-phosphate (S1P) is released at sites of tissue injury and effects cellular responses through activation of G protein-coupled receptors. The role of S1P in regulating cardiomyocyte survival following in vivo myocardial ischemia-reperfusion (I/R) injury was examined by using mice in which specific S1P receptor subtypes were deleted. Mice lacking either S1P2 or S1P3 receptors and subjected to 1-h coronary occlusion followed by 2 h of reperfusion developed infarcts equivalent to those of wild-type (WT) mice. However, in S1P2,3 receptor double-knockout mice, infarct size following I/R was increased by &gt;50%. I/R leads to activation of ERK, JNK, and p38 MAP kinases; however, these responses were not diminished in S1P2,3 receptor knockout compared with WT mice. In contrast, activation of Akt in response to I/R was markedly attenuated in S1P2,3 receptor knockout mice. Neither S1P2 nor S1P3 receptor deletion alone impaired I/R-induced Akt activation, which suggests redundant signaling through these receptors and is consistent with the finding that deletion of either receptor alone did not increase I/R injury. The involvement of cardiomyocytes in S1P2 and S1P3 receptor-mediated activation of Akt was tested by using cells from WT and S1P receptor knockout hearts. Akt was activated by S1P, and this was modestly diminished in cardiomyocytes from S1P2 or S1P3 receptor knockout mice and completely abolished in the S1P2,3 receptor double-knockout cardiomyocytes. Our data demonstrate that activation of S1P2 and S1P3 receptors plays a significant role in protecting cardiomyocytes from I/R damage in vivo and implicate the release of S1P and S1P2 and S1P3 receptor-mediated Akt activation in this process.

cardioprotection; mitogen-activated kinase; G protein-coupled receptors; infarct

Sphingosine 1-phosphate (S1P) is a bioactive lysosphospholipid generated through the breakdown of sphingomyelin. A number of regulated enzymes, including sphingomyelinase and sphingosine kinase, control its formation (40). A role for S1P in regulating cellular responses to injury and inflammation has become increasingly well accepted. In the heart, as in other tissues, sphingomyelinase is activated by ischemia-reperfusion (I/R) (anoxia-reoxygenation) and by cytokines such as TNF-α, suggesting that sphingolipid metabolites (ceramide, sphingosine, and S1P) are generated and may participate in cellular responses to these interventions (5, 8, 12, 23). Sphingosine kinase has also been shown to be activated by I/R in the heart (18). Although intracellular actions of sphingomyelin metabolites had been examined for many years, the cloning of G protein-coupled receptors with specificity for S1P led to recognition that sphingolipid-mediated responses are effected, in large part, through extracellular activation of cell surface receptors (6, 16, 26).

The S1P receptors, originally classified into the edg receptor family, are now referred to as S1P1–S1P5. The S1P1 (edg1), S1P2 (edg5), and S1P3 (edg3) receptors are ubiquitously expressed, whereas the expression of S1P4 and S1P5 receptors is more restricted. The selectivity in coupling of these receptors to specific G proteins and signal-transduction pathways has not been well established because few receptor subtype-selective agonists or antagonists are available. The generation of knockout mice, in which specific S1P receptor genes are deleted by homologous recombination (2, 15, 17, 28), has therefore provided a much-needed means for examining the roles of the different S1P receptor subtypes as well as their downstream targets.

S1P1 receptor knockout mice (S1P1−/− mice) show embryonic lethality due to the aberrant vasculogenesis that results from loss of S1P receptors in vascular endothelial cells (2, 28). In contrast S1P2, S1P3, or S1P2,3 receptor knockout mice (S1P2−/−, S1P3−/−, or S1P2,3−/−) are viable and show only modest phenotypic changes (15, 17). Our previous studies examining mouse embryonic fibroblasts (MEF cells) derived from these mice revealed that PLC activation is regulated by S1P3 receptors alone, Rho activation is regulated by both S1P2 and S1P3 receptors, and adenylate cyclase inhibition is regulated by S1P1 receptors, because this response is not lost in MEF cells from S1P2−/−, S1P3−/−, or S1P2,3−/− mice (15, 17).

Sphingolipid metabolites such as S1P and ceramide have been suggested to regulate cell survival. Whereas ceramide is considered to be proapoptotic, S1P can suppress ceramide-mediated apoptosis, providing a yin-yang aspect to sphingomyelinase signaling (9). S1P has been shown to activate Akt (14, 37, 39), which has been associated with cell survival in cardiomyocytes (10, 31, 38). In addition, S1P has been shown to protect neonatal rat cardiomyocytes and perfused rabbit and mouse hearts from ischemic damage (5, 18, 19, 22). However, neither the receptor subtype nor the signal-transduction pathways mediating these effects has been established, nor has an in vivo protective role for endogenously released S1P been demonstrated. Accordingly, we designed experiments to examine the cell-survival pathways regulated by S1P in cardiomyo-
ocytes, to determine whether S1P receptor activation participated in the response to I/R injury in vivo, and to identify the S1P receptor subtypes and downstream mediators affording cardioprotection.

The experiments reported here demonstrate that I/R injury is not altered in either S1P2−/− or S1P3−/− mice but is markedly increased in S1P2,3−/− mice. Examination of the signal-transduction pathways regulated by S1P in isolated cardiomyocytes and by I/R in WT and S1P receptor knockout mice revealed that activation of Akt in cardiomyocytes, although modestly diminished in S1P2−/− and S1P3−/− mice, is severely compromised in cardiomyocytes and in vivo from hearts from S1P2,3−/− mice. We conclude that S1P formation during I/R limits cardiomyocyte damage by stimulating both S1P2 and S1P3 receptors and suggest that the protective effect of S1P2,3 receptor stimulation occurs through activation of Akt-mediated survival pathways.

**EXPERIMENTAL PROCEDURES**

*Animals.* Generation and maintenance of S1P2−/−, S1P3−/−, and S1P2,3−/− mice were previously reported (15, 17). Animals had free access to water and food. All experiments reported here were performed using 24- to 28-wk-old (25–35 g) mice of either sex. Wild-type (WT) littermate animals were used as controls for all experiments with S1P2−/− or S1P3−/− mice. For experiments with S1P2,3−/− mice, the low frequency of obtaining double-knockout mice (1/16) and WT mice (1/16) from the same litter (1/256) necessitated the use of age-matched WT mice of the same background as controls. All procedures were performed in accordance with the National Institutes of Health/University of California, San Diego Guide for the Care and Use of Laboratory Animals and approved by the UCSD Institutional Animal Care and Use Committee.

*RT-PCR.* Cultured adult mouse cardiomyocytes and whole mouse hearts were collected and processed by methods described in previous studies (1, 45). Total RNA was isolated using RNeasy (Qiagen), and cDNA was produced by using Superscript reverse transcriptase (Invitrogen). PCR was performed with an initial denaturation step at 94°C for 5 min followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 2 min and a final extension step at 72°C for 10 min. The following S1P receptor-specific primers were used for PCR amplification: S1P1, 5′-CTCTGTCGCAAGGCAATGTA-3′; S1P2, 5′-GAATGGACCTCTGGTTAGGTG-3′; S1P3, 5′-ATGGGCCAGCCTTGTACTCGGAG-3′; S1P4, 5′-GAGTGAGCTTGTAGGTGGTG-3′; and S1P5, 5′-ATCTGTGCGCTCTATGCAAGGA-3′. Equal amounts of total protein were loaded. The antibodies used for immunoblotting were as follows: rabbit anti-phospho-Akt (Ser473), rabbit total Akt, rabbit anti-phospho-ERK1/2, rabbit total ERK1/2, rabbit anti-phospho-p38, rabbit total p38, mouse anti-phospho-JNK, or rabbit total JNK (Cell Signaling Technology).

*RT-PCR.*** Cultured adult mouse cardiomyocytes and whole mouse hearts were collected and processed by methods described previously (45). Total RNA was isolated using RNeasy (Qiagen), and cDNA was produced by using Superscript reverse transcriptase (Invitrogen). PCR was performed with an initial denaturation step at 94°C for 5 min followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 2 min and a final extension step at 72°C for 10 min. The following S1P receptor-specific primers were used for PCR amplification: S1P1, 5′-CTCTGTCGCAAGGCAATGTA-3′; S1P2, 5′-GAATGGACCTCTGGTTAGGTG-3′; S1P3, 5′-ATGGGCCAGCCTTGTACTCGGAG-3′; S1P4, 5′-GAGTGAGCTTGTAGGTGGTG-3′; and S1P5, 5′-ATCTGTGCGCTCTATGCAAGGA-3′. Equal amounts of total protein were loaded. The antibodies used for immunoblotting were as follows: rabbit anti-phospho-Akt (Ser473), rabbit total Akt, rabbit anti-phospho-ERK1/2, rabbit total ERK1/2, rabbit anti-phospho-p38, rabbit total p38, mouse anti-phospho-JNK, or rabbit total JNK (Cell Signaling Technology).

*Immunoblot analysis.* For Western blotting, adult mouse cardiomyocytes or cardiac homogenates were prepared as described previously (45). Equal amounts of total protein were loaded. The antibodies used for immunoblotting were as follows: rabbit anti-phospho-Akt (Ser473), rabbit total Akt, rabbit anti-phospho-ERK1/2, rabbit total ERK1/2, rabbit anti-phospho-p38, rabbit total p38, mouse anti-phospho-JNK, or rabbit total JNK (Cell Signaling Technology).

*Animal model.* Occlusion and reperfusion of the coronary artery were performed as previously reported (44). Briefly, mice were anesthetized with an intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine (5 mg/kg) and were placed in a supine position under body-temperature control. Each animal was endotracheally intubated and ventilated with a tidal volume of 0.5 ml at a rate of 120 strokes/min by using a rodent respirator (model no. 683; Harvard Apparatus). After left thoracotomy, a 7-0 surgical suture was passed underneath the left anterior descending coronary artery (LAD) at a position 2 mm from the tip of the left auricle under the aid of a stereoscope (Nikon). PE-10 tubing (1–2 mm in length) was placed along the vessel as a cushion and was secured around the tubing to occlude the LAD. For the sham-operated control mice, the procedure was performed as above except that the suture was not secured around the LAD to occlude the vessel. Myocardial ischemia was verified by blanching of the left ventricle (LV) and by change in electrocardiogram. Blood flow was restored after 1 h of occlusion by removing the ligature and PE tubing.

*Assessment of area at risk and infarct size.* Following 2 h of reperfusion, the LAD was reoccluded and 5% Evans blue dye (0.2 ml) was injected into the LV cavity with a 27-gauge needle to define the nonischemic zone. The heart was excised immediately and rinsed in saline to remove excess dye, and the LV was frozen and cut transversely into five slices of equal thickness. These samples were incubated in 1% 2,3,5-triphenyltetrazolium chloride-containing Tris-HCl buffer (pH 7.8) at 37°C for 10 min to stain the viable myocardium (brick red) and then were fixed in 10% formalin-phosphate buffered saline for 24 h. Each slice was weighed and photographed from both sides by using a microscope equipped with a high-resolution digital camera (COOLPIX 990; Nikon). The area at risk (AAR), infarcted
RESULTS

*S1P receptor expression.* RT-PCR analysis was used to determine the pattern of expression of S1P receptors in adult mouse cardiomyocytes and in mouse heart. Transcripts of the S1P1, S1P2, S1P3, and S1P5 receptors were detected in both isolated cardiomyocytes and the whole adult heart (Fig. 1). S1P4 receptors were not detected in either cardiac preparation, although S1P4 receptor transcripts were detected in other tissues using the same primers (data not shown).

*S1P2 and S1P3 receptors mediate protection from I/R injury.* Adult S1P2/−/−, S1P3/−/−, and S1P2,3/−/− mice are phenotypically normal, although defects in S1P-mediated cellular signaling have been demonstrated in MEF cells isolated from these animals (15, 17). To determine whether S1P receptors play a role in the response to I/R injury in vivo, we compared WT and S1P receptor-null mice after in vivo I/R by using a previously established model (44). Cardiomyocyte cell death in hearts exposed to 1 h of coronary occlusion followed by 2 h of reperfusion was assessed by using 2,3,5-triphenyltetrazolium chloride staining (described in Experimental Procedures). A representative photomicrograph of a short-axis section from a WT mouse LV is shown in Fig. 2A, and the areas quantified to assess ischemic injury are delineated. Evans blue dye-positive areas represent nonischemic tissue, whereas the ischemic area (the AAR) is comprised of the white infarcted necrotic tissue (I) plus the red viable salvaged tissue (2).

We first compared WT and S1P3/−/− mice. The severity of the ischemic insult was not different in the two groups based on the similar values for AAR expressed relative to total LV mass (Fig. 2B). The infarct size, reflective of the amount of nonviable myocardium, was also not significantly different between S1P3/−/− and WT mice, whether expressed relative to AAR or total LV mass (Fig. 2B). We subsequently compared WT and S1P2/−/− mice. The severity of the insult was not significantly different between these two groups, as seen by the AAR relative to LV. As observed for S1P3/−/− mice, the size of the infarct relative to either AAR or LV was not significantly different between S1P2/−/− and WT mice (Fig. 3). These data indicate that the loss of either the S1P2 or S1P3 receptor alone does not alter the in vivo response to I/R injury.

The S1P2 and S1P3 receptors could serve redundant functions by coupling to common downstream pathways. Accordingly, we further tested the involvement of S1P receptors in ischemic injury by examining the response to myocardial I/R injury in S1P2,3/−/− mice. As shown in Fig. 4, the areas at risk were not different in WT and S1P2,3/−/− mice. Importantly, however, infarct size (expressed as a percentage of AAR) was increased by >50% in S1P2,3/−/− compared with WT mice (Fig. 4). Infarct size expressed relative to LV mass was also significantly elevated. Thus combined activation of S1P2 and S1P3 receptors provides a protective signal during in vivo I/R that is lost in S1P2,3/−/− mice.

To rule out the possibility that the protective role of S1P receptors is due to S1P receptor-mediated effects on heart rate [through activation of potassium currents (13, 41)], heart rate was monitored by continuous electrocardiographic recording throughout the period of I/R. No differences in heart rate were observed among the groups of mice examined (data not shown), indicating that differences in chronotropic responsiveness do not underlie the altered susceptibility to injury.
MAP kinase activation pathways are not altered in S1P2,3−/− mice subjected to in vivo I/R. To examine the possible role of MAP kinase activation in the protective effects of S1P receptors, we first characterized the kinetics of activation of various MAP kinases following I/R in WT mice. Phosphorylation of p38, ERK, and JNK MAP kinases was examined by Western blotting with phospho-specific antibodies. Both ischemia and subsequent reperfusion led to increased p38 phosphorylation (Fig. 5A), as previously observed in isolated rat and rabbit hearts (4, 29). Phosphorylation of ERK and JNK was not significantly increased during ischemia but increased following reperfusion, with the peak of activation occurring after 15 min of reperfusion (Fig. 5A), consistent with previous findings from isolated rat and rabbit heart (4, 36). To determine whether altered activation of these MAP kinases could be responsible for the differential susceptibility to I/R injury, the phosphorylation states of ERK, JNK, and p38 MAP kinases were compared in S1P2,3−/− mice subject to ischemia and 15 min of reperfusion. There was no significant difference in the magnitude of reperfusion-induced phosphorylation of any of the MAP kinases in the S1P2,3−/− vs. WT mice (Fig. 5B). Thus MAP kinase signaling during in vivo I/R is not compromised in the combined absence of the S1P2 and S1P3 receptors.

I/R-induced Akt activation in S1P receptor knockout mice. Similar experiments were then carried out examining Akt activation in response to I/R. Western blotting to detect Akt phosphorylation at Ser473 in the catalytic loop revealed that Akt phosphorylation increases during reperfusion following ischemia, consistent with previous studies carried out on isolated, perfused rat hearts (20, 43). In the WT mouse heart the increase in Akt phosphorylation was maximal at 15 min of reperfusion (Fig. 6A). Akt phosphorylation was then compared in WT and S1P2,3−/− mouse hearts following I/R. The fivefold increase in phospho-Akt observed in WT mice was markedly attenuated (by ~70%) in the S1P2,3−/− mice (Fig. 6B). These data indicate that a significant component of the Akt activation observed during I/R occurs through S1P2 and S1P3 receptor activation and suggest that endogenously released S1P may serve to protect against I/R injury through this pathway.

To further establish a relationship between Akt activation and the protective effect of S1P receptor activation, we tested S1P2−/− or S1P3−/− mice, neither of which showed altered infarct size in response to I/R. Mice from both lines were subjected to I/R, and Akt activation was assessed. In contrast to what we observed for the S1P2,3−/− mice, Akt activation by I/R in either S1P2−/− or S1P3−/− mice was not significantly different from that of WT mice (Fig. 7).
S1P-mediated Akt activation in WT and S1P receptor knock-out adult mouse cardiomyocytes. The data above indicate that Akt activation after in vivo I/R correlates with S1P-mediated protection. Although the heart is largely myocytes, other endogenous or invading cells responsive to S1P (e.g., endothelial cells, macrophages) could be present. To demonstrate that the alterations observed in vivo reflect the response of cardiomyocytes to S1P, we isolated cardiomyocytes from WT, S1P2/−/−, S1P3/−/−, and S1P2,3/−/− mice and assessed the ability of S1P to activate Akt. Treatment of WT adult mouse myocytes with 5 μM S1P (Avanti Polar Lipids) significantly increased Akt phosphorylation (Fig. 8). Although the response was less robust than that elicited by I/R, the pattern observed was similar. S1P induced a smaller and not statistically significant increase in Akt activation in cardiomyocytes from S1P2/−/− or S1P3/−/− mice, whereas deletion of both S1P2 and S1P3 receptors resulted in a complete loss of S1P-mediated Akt activation.

**DISCUSSION**

Sphingosine is released from, and S1P is formed in, isolated rabbit hearts subject to hypoxia and acidosis (5). The addition of S1P to neonatal rat ventricular myocytes has been demonstrated to confer cardioprotection against hypoxia (22), and S1P also protects against global I/R damage in isolated mouse hearts (19). A role for S1P in conferring ischemic preconditioning in the isolated heart has also been suggested (24). Activation of sphingosine kinase, the upstream kinase responsible for producing S1P, has more recently been suggested to protect the isolated perfused heart from I/R damage (18). Our findings provide the first in vivo evidence that G protein-coupled S1P2 and S1P3 receptors are stimulated during I/R and promote cardiomyocyte survival.

The data presented here also provide insight into the signaling pathways by which S1P can affect cardioprotection in vivo. As demonstrated here, the extent of I/R damage did not differ...
in the $S1P_3^{-/-}$ vs. WT mice. According to our previously published studies (15), there is nearly complete loss of S1P-mediated phosphoinositide hydrolysis in MEF cells from $S1P_3^{-/-}$ mice, and we also observed complete loss of S1P-stimulated phosphoinositide hydrolysis in myocytes isolated from $S1P_3^{-/-}$ hearts (data not shown). Thus if elevated S1P elicits PLC activation and generation of its downstream second messengers through $S1P_3$ receptors in the ischemic myocardium, these responses do not appear to be required for S1P-mediated protection.

MAP kinase pathways have also been implicated in control of cell survival in the myocardium. In vivo, all three MAP kinase pathways (ERK, JNK, and p38) are activated by reperfusion following ischemia (Fig. 5) (7). However, neither ERK, JNK, nor p38 activation by I/R is impaired in the $S1P_2,3^{-/-}$ mice. This finding suggests that the major pathways leading to MAP kinase activation in I/R are not initiated through stimulation of the $S1P_2$ or $S1P_3$ receptors. Thus activation of another receptor likely contributes to the activation of MAP kinases in vivo I/R. In addition, the observation that infarct size is significantly increased in $S1P_2,3^{-/-}$ mice, even in the face of unaltered MAP kinase activation, indicates that activation of MAP kinases is not sufficient to support cardiomyocyte survival.

The phosphorylation of Akt that accompanies I/R is, in contrast, markedly attenuated in the $S1P_2,3^{-/-}$ mice. Smaller and insignificant decreases are affected by loss of either $S1P_2$ or $S1P_3$ receptors alone. Thus stimulation of both $S1P_2$ and $S1P_3$ receptors appears to contribute to activation of Akt in vivo. The redundant or overlapping functions of these receptors in coupling S1P actions to phosphorylation of Akt is also seen in our in vitro studies on isolated cardiomyocytes. Cardiomyocytes lacking either the $S1P_2$ or $S1P_3$ receptor demonstrate a partial loss in Akt phosphorylation, whereas there is complete loss of S1P-mediated phosphorylation of Akt in $S1P_2,3^{-/-}$ cardiomyocytes. That either $S1P_2$ or $S1P_3$ receptors can mediate Akt activation and concomitant cardioprotection further explains why I/R damage is not aggravated in mice lacking only $S1P_2$ or only $S1P_3$ receptors.
A surprising aspect of our studies is that the S1P1 receptor, still present in the S1P2,3/−/− mice, does not confer greater protection against I/R injury. Akt activation by I/R is markedly diminished in the S1P2,3/−/− mice despite the presence of the S1P1 receptor, shown in other systems to couple to Akt activation (3, 25, 33). The S1P1 receptor in adult mouse cardiomyocytes also couples poorly to this pathway, because no Akt activation is observed in cells from S1P2,3/−/− mice. The reason that the S1P2 and S1P3 receptors, but not the S1P1 receptor, regulate Akt activation in cardiomyocytes is under study.

Akt is a well-established mediator of cardioprotection in I/R injury both in vitro and in vivo, as demonstrated by transfection, gene delivery, and transgenic approaches (10, 30–32). Mechanisms of Akt-mediated cardioprotection are under intense investigation. Akt has been shown to increase endothelial NO synthase (eNOS) phosphorylation, and a role for NO in protection against ischemic damage is suggested by experiments with eNOS knockout mice (11, 21). In endothelial cells, S1P activates eNOS via an Akt-mediated pathway, and this occurs via the S1P3 receptor (34). More recently, it has been reported (42) that administration of exogenous S1P is able to protect the heart from I/R injury via this S1P3 receptor-mediated pathway. Interestingly, our data indicate that in cardiomyocytes, Akt activation occurs predominantly through the S1P2 receptor and to a lesser extent through the S1P3 receptor. In vivo, we find that both the S1P2 and S1P3 receptors contribute to I/R-induced Akt activation and that an increase in ischemic damage is not seen unless both S1P2 and S1P3 receptors are deleted. Because the Akt activation in response to in vivo I/R is greater than the Akt activation seen in isolated cardiomyocytes, it is likely that other cell types (e.g., vascular endothelial cells or fibroblasts) or other activators of Akt are contributing to this response. As a working hypothesis, we suggest that the protective effects of S1P released in response to I/R involve S1P3 receptor activation of eNOS, via Akt, in cardiomyocytes, it is likely that other cell types (e.g., vascular endothelial cells or fibroblasts) or other activators of Akt are contributing to this response. As a working hypothesis, we suggest that the protective effects of S1P released in response to I/R involve S1P3 receptor activation of eNOS, via Akt, in cardiomyocytes, as well as S1P2 and S1P3 receptor activation of Akt in cardiomyocytes.

In conclusion, our findings indicate that S1P activation of its cognate G protein-coupled receptor on cardiomyocytes serves as a signal for Akt activation and cardiomyocyte protection during I/R in vivo. Subtype-selective agonists for S1P2 or S1P3 receptors could therefore be novel therapeutic modalities for limiting the extent of cardiomyocyte loss associated with acute I/R injury in the heart.

ACKNOWLEDGMENTS

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GRANTS

This work was supported by National Institutes of Health grants HL-46345, (to J. H. Brown), HL-28143 (to J. H. Brown), HL-43026 (to J. H. Omens), MH-56199 (to J. Chun), and MH-01723 (to J. Chun) and by an American Heart Association postdoctoral fellowship (to T. Zhang) and an American Heart Association predoctoral fellowship (to C. K. Means).

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