Redox Factor-1 (Ref-1) Mediates the Activation of AP-1 in HeLa and NIH 3T3 Cells in Response to Heat Shock*

(Received for publication, February 19, 1999, and in revised form, March 29, 1999)

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The early response genes, c-Fos and c-Jun, are induced by environmental stress and are thought to modulate injury processes via the induction of AP-1-dependent target genes. AP-1 activation is thought to be regulated by changes in intracellular oxidation/reduction reactions involving the redox factor-1 (Ref-1) protein. In this study, NIH 3T3 and HeLa cells were used to determine whether heat shock induces the AP-1 transcription factor via signaling pathways involving Ref-1. Reverse transcriptase-polymerase chain reaction analysis and immunoblotting demonstrated that c-Fos and c-Jun were induced 2-10 h following heat shock, and this induction was accompanied by an increase in AP-1 DNA binding. Electrophoretic mobility shift assay extracts immunodepleted of Ref-1 protein demonstrated that the increase in AP-1 DNA-binding activity following heating was dependent upon the presence of Ref-1 and that Ref-1 regulates inducible, but not basal, AP-1 DNA-binding activity. This was confirmed by the restoration of heat-inducible DNA binding upon addition of Ref-1 to immunodepleted extracts. The ability of Ref-1 from heated cells to stimulate AP-1 DNA binding was abolished by chemical oxidation and restored by chemical reduction. These results indicate that heat shock activates c-Fos/c-Jun gene expression and AP-1 DNA binding and suggests that redox-sensitive signal transduction pathways involving Ref-1 may mediate heat-induced alterations in AP-1 activation.

Most living cells are sensitive to sudden elevations in temperature and respond to this environmental insult by activating the heat shock response (1, 2). The heat shock response has been intensively investigated and is primarily mediated at the transcriptional level by pre-existing transcriptional activators, termed heat shock factors (HSFs)\(^\text{3}\) (3). Once activated, HSFs bind to regulatory heat shock elements present in the promoter region of genes coding for heat shock proteins (HSPs) (1, 3). In addition to HSFs, it appears that several additional signal transduction cascades are also activated in response to heat including p38/HOG1 kinase (4), Jun N-terminal kinase (5), MAPK1 (6), and protein kinase C (7–9). These signal pathways are also activated by a wide variety of additional environmental insults including oxidative stress. Studies of cross-resistance induced in response to oxidative stress and heat shock (10, 11) suggest that one aspect of the cellular response to heat may be similar to cellular responses to oxidative stress.

The mechanism responsible for causing cell death after thermal injury remains unclear. It has been suggested that one aspect of heat-induced cellular injury results from mitochondrial damage that disrupts intracellular oxidation/reduction reactions (10–17). Hence, heat shock may result in altered generation of reactive oxygen species as well as alterations in intracellular antioxidant capacity (13, 15, 17–20). Mammalian cells and tissues exposed to heat shock have been shown: 1) increased conversion of xanthine dehydrogenase to the superoxide-generating enzyme xanthine oxidase, 2) alterations in thiol metabolism leading to the increased synthesis of glutathione (GSH) and increase in formation of oxidized glutathione (GSSG), 3) increased sensitivity to heat-induced cell killing when GSH is depleted, and 4) increased resistance to heat-induced cell killing in stable H2O2-resistant cell types which overexpress cellular antioxidants (glutathione, catalase, superoxide dismutase, glutathione peroxidase, and glutathione transferase) (13, 15, 16, 21, 22). In addition, in Saccharomyces cerevisiae prooxidant production, as measured by the oxidation-sensitive fluorescent probe, 2′,7′-dichlorofluoroscin diacetate, increased following heat shock (17). Manipulations designed to reduce the production of reactive oxygen species (over expression of catalase, superoxide dismutase, cytochrome c peroxidase, and anaerobic conditions) protected cells from the lethal effects of heat (17). These studies support the hypothesis that heat shock induces an imbalance in intracellular oxidation/reduction (redox) reactions resulting in increased steady-state prooxidant production and oxidative stress that contributes to the biological effects of heat shock.

Alterations in intracellular oxidation/reduction reactions have been shown to activate signal transduction cascades that regulate as early response genes. These genes are believed to function in a protective or reparative capacity (23–25). The early response genes c-Fos and c-Jun are members of a multigene family implicated in a number of stress-induced signal transduction cascades and thus provide useful models for investigating stress-invoked alterations in gene expression (26). c-Fos and c-Jun proteins associate in homo- and/or heterodimers to form the mammalian transcription factor AP-1. It has been suggested that AP-1 may regulate expression of downstream target genes that are known to be involved with cellular antioxidant defense mechanisms (27–32). Stress-induced activation of these early response genes appears to rely, at least in part, on changes in intracellular oxidation/reduction (redox)
(33–35). Hence, mammalian cells appear to capitalize on inherent redox-sensitive signaling circuitry to respond to certain forms of environmental stresses that perturb oxidative metabolism (27, 36).

At least one mechanism regulating c-Fos/c-Jun DNA binding is mediated by a conserved cysteine (Cys) located in the basic DNA-binding domain of both proteins (27). In vitro these regulatory cysteines are not permissive for DNA binding under oxidized conditions, whereas reduction to a sulfhydryl state promotes DNA binding (27, 35). As such, these critical cysteines act as a redox-sensitive “sulfhydryl switch” that reversibly modulates DNA binding (36). In the absence of reducing agents, the redox factor-1 (Ref-1) protein regulates c-Fos/c-Jun DNA binding via the same conserved cysteine. Ref-1 (also designated ARP, and HAP-1) is a DNA repair enzyme that also acts as a signaling factor regulating the DNA binding of several redox-sensitive transcription factors including NF-kB, Egr-1, and p53 (35, 36). Interestingly, a wide range of cellular stress agents also induce these transcription factors (23, 24). Thus, Ref-1 may act as a pivotal signaling factor involved in the induction of early response genes, such as c-Fos and c-Jun.

It has been suggested heat shock causes perturbations in cellular redox status. In addition, several early response genes as well as signaling cascades are activated by changes in cellular redox conditions. Hence, it is reasonable to hypothesize that heat-induced increases in early response gene expression (c-Fos and c-Jun) leading to increased AP-1-DNA binding may encompass a mechanism involving Ref-1. To address this hypothesis, the expression of c-Fos and c-Jun as well as the activation of the AP-1 transcription factor was determined following heat shock in human and rodent cell lines. When c-Fos and c-Jun expression as well as AP-1 activation were found to increase following heat shock, the role of Ref-1 in the activation of AP-1 binding was determined. These results indicate the induction of the early response genes c-Fos and c-Jun in response to heat shock is similar to that observed for other types of environmental stress. Furthermore, the redox-sensitive signal transduction protein, Ref-1, via heat-induced alterations in the oxidation/reduction state of the protein appears to regulate heat-inducible, but not basal, AP-1 DNA-binding activity. These results suggest that early response genes may play a role in the cellular responses to heat shock and that redox-sensitive signal transduction pathways involving Ref-1 may represent a common mechanism for the induction of AP-1 binding activity in response to environmental stress.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Heat Shock Conditions**—HeLa (human cervical carcinoma) and NIH 3T3 (rodent fibroblasts) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated calf serum with penicillin (50 units/ml) and streptomycin (50 units/ml) in a humidified 5% CO₂ atmosphere at 37 °C. For heat shock experiments, cells were seeded at 2.5 × 10⁵ cells/10-cm diameter plate the night before exposure. Cells were heated at various time points by submerging parafilm sealed 10-cm plates in a pre-warmed circulating water bath at 45 °C for 15 min. Cells were immediately placed at 37 °C after heating and harvested at various time points.

**mRNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis**—Total cellular RNA was isolated by using TRI reagent (Molecular Research, Cincinnati, OH) following the protocol of the manufacturer. The RNA was checked for purity and used in a quantitative RT-PCR analysis method that has been previously described (37). Primer sequences for c-Fos, c-Jun, and glyceraldehyde-3-phosphate dehydrogenase were selected on separate exons to distinguish amplified products of mRNA from possible contaminating DNA or precursor RNA. The primer sequences and PCR product sizes have been previously described (37). Five microliters of the PCR amplified products were analyzed by electrophoresis on a 1% agarose gel. Gels were stained with ethidium bromide, photographed, dried and exposed to phosphoimager screen for quantitation of incorporated radioactivity in each individual band. Quantitation of results was obtained using a STORM 840 Phosphorimager. All results are presented as fold increase above baseline control, unheated cells.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis**—Whole cell extracts were prepared at 2, 4, 6, 8, 10, and 24 h after heating as described (38). Proteins in samples were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose filter using a semi-dry transfer apparatus (Owl, Inc). Western blotting analysis was done with polyclonal antibodies to c-Fos (Oncogene Science, AP1), c-Jun (Oncogene Science, AP2), and Ref-1 (Santa Cruz Biotech, Inc). Antibodies were diluted 1:1000 in a 2.5% milk, phosphate-buffered saline, solution. Protein bands were visualized with a chemiluminescence method (Amersham Pharmacia Biotech).

**Electromobility Shift Assays**—EMSA were performed as described previously utilizing a [32P]-radiolabeled oligonucleotide corresponding to the consensus AP-1 DNA-binding site (38). Briefly, whole cell extracts (10 µg) were incubated with poly(dI-dC) for 10 min on ice, followed by addition of radiolabeled oligonucleotide (200,000 cpm of radiolabeled probe per reaction) and incubated at 25 °C for 20 min. Supershift experiments were performed by the addition of either anti-Fos or anti-Jun antibody to the whole cell protein extract-poly(dI-dC), radiolabeled oligonucleotide followed by incubation for 20 min at 25 °C. For the cold AP-1 oligomer competition experiment, 1 µg of unlabeled AP-1 oligomer was added to the whole cell extracts prior to electrophoresis-poly(dI-dC) complex, kept on ice for 10 min followed by addition of radiolabeled oligonucleotide and incubation for 20 min at 25 °C. Samples were run on a 4.5% nondenaturing polyacrylamide gel electrophoresis, dried, and exposed to the phosphoimager screen for quantitation using a STORM 840 Phosphorimager from Molecular Dynamics.

**Immunodepletion EMSAs** for Ref-1 were performed immediately after the extracts were prepared by adding 5 µl of anti-Ref-1 and 25 µl of Protein A to 20 µl of EMSA control and heated extracts as described (40). After 2 h of gentle shaking at 4 °C, the tubes were spun at 12,000 rpm for 1 min and washed. The protein levels in the remaining extracts were determined and EMSAs were performed. Chemical modification of immunoprecipitated Ref-1 was performed by adding either diamide (80 mM), N-ethylmaleimide (NEM) (50 µM), or dithiothreitol (DTT) (8 mM) to immunoprecipitation/protein A pellet for 30 min. To remove the free residual chemicals from the immunoprecipitation reaction, the pellets were washed eight times before EMSA using non-heated Ref-1 immunodepleted extracts.

**RESULTS**

**Induction of Fos and Jun in Response to Heat**—Previous investigations have shown that HeLa cell c-Jun mRNA levels increase in response to heat (41). As a first step, therefore, we sought to confirm and expand upon this finding by examining c-Jun and c-Fos mRNA levels following heating. Experiments were performed with NIH 3T3 and HeLa cells, representing immortalized and fully transformed cell lines, respectively. Asynchronous cycling NIH 3T3 and HeLa cells were heated (45 °C for 15 min) and then incubated at 37 °C until RNA isolation at 2, 4, 6, 8, 10, and 24 h. c-Fos and c-Jun mRNA levels were determined using quantitative RT-PCR (37). In NIH 3T3 cells (Fig. 1A), c-Fos and c-Jun expression increased 6- and 3-fold, respectively, when compared with control, unheated cells. The induction was initially apparent 2 h following heating and returned to basal levels by 10 h. Similarly, in HeLa cells (Fig. 1B), c-Fos and c-Jun expression was induced 5- and 3-fold, respectively, following heating. Thus, in NIH 3T3 and HeLa cells the relative abundance of c-Fos and c-Jun mRNA is increased in response to heat shock. Furthermore, the accumulation of c-Fos and c-Jun mRNA following heat shock is similar in both magnitude and temporal expression to other cellular proteins.

To determine whether the increased abundance of c-Fos and c-Jun mRNA in response to heat shock is accompanied by increased accumulation of immunoreactive protein, total cellular protein was isolated from both cell lines at 2, 4, 6, 8, 10, and 24 h following heat shock. Following heat shock in NIH 3T3 cells, an increase in c-Fos and c-Jun immunoreactive protein was apparent at 4–6 h (Fig. 2A), reached a peak at 8 h (5- and 6-fold, respectively, as compared with control unheated cells),
and returned to base line by 24 h. Similarly, in HeLa cells an increase in c-Fos and c-Jun immunoreactive protein was demonstrated 4–6 h following heat shock and returned to base line by 24 h. Similarly, in HeLa cells an increase in c-Fos and c-Jun immunoreactive protein was demonstrated 4–6 h following heat shock and returned to base line by 24 h (Fig. 2).

**FIG. 1.** Increased expression of c-Fos and c-Jun mRNA in response to heat. RT-PCR analysis of RNA isolated from NIH 3T3 (A) and HeLa (B) cells subjected to heat stress. HeLa or NIH 3T3 cells were plated at 2.5 × 10^6 cells/10-cm diameter plate, heated to 45 °C for 15 min, and then incubated at 37 °C until harvested. RNA was isolated from non-heated or heated cells at 2, 4, 6, 8, 10, and 24 h following heat shock. Relative mRNA level of each target gene was first normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase mRNA level in individual samples, and the fold increase was determined relative to non-heated cells. C, control.

**FIG. 2.** Increased expression of c-Fos and c-Jun protein in response to heat. Western blot analysis of whole cell extracts from cells subjected to heat stress. Total cellular protein was isolated from asynchronously cycling NIH 3T3 (A) and HeLa (B) cells from control, non-heated (lane 1) and heated cells at 2, 4, 6, 8, 10, and 24 h. Cells were heated at 45 °C for 15 min. 10 μg of cellular protein was separated by SDS-PAGE, transferred onto nitrocellulose, and processed for immunoblotting with rabbit polyclonal antibodies to c-Fos or c-Jun (Oncogene Products Research). All results are presented as fold increase above base-line control, unheated cells. Equal protein loading was determined using a Bradford protein assay. C, control.

and returned to base line by 24 h. Similarly, in HeLa cells an increase in c-Fos and c-Jun immunoreactive protein was demonstrated 4–6 h following heat shock and returned to base line by 24 h (Fig. 2B). Thus, increased accumulation of c-Jun and c-Fos mRNA following heat shock correlated with an increase in protein levels of similar magnitude and temporal expression. Furthermore, these experiments demonstrate that heat, like other types of environmental stress, increases early response gene expression.

**Induction of AP-1 DNA-binding Activity following Heating—** The Fos and Jun protein families form an array of heterodimeric protein complexes that bind to specific cis-acting DNA regulatory elements, referred to as AP-1 sites, to activate the expression of downstream target genes (24, 42). AP-1 sites are present in the promoter region of many genes, including c-Fos and c-Jun, as well as in other genes involved in the cellular response to environmental stress (34). Since c-Fos and c-Jun comprise the AP-1 DNA-binding complex and both c-Fos and c-Jun expression increase in response to heat, we sought to determine whether AP-1 DNA-binding activity increased following heat exposure.

AP-1 DNA binding was measured by performing EMSA with extracts from NIH 3T3 and HeLa cells. Cells were heated to 45 °C for 15 min and then incubated for 2, 4, 6, 8, 10, and 24 h at 37 °C until harvested. NIH 3T3 cells demonstrated no difference in AP-1 DNA-binding activity between unheated controls and cells harvested at 2 and 4 h after heating (Fig. 3A, lanes 1–4). In contrast, a 2-fold increase in DNA-binding activity was noted at 6 h (lane 5), reached a peak induction of 3-fold at 8 and 10 h (lanes 6 and 7), and returned to base line at 24 h (lane 8). These experiments were repeated in HeLa cells (Fig. 3B) with a similar induction of AP-1 binding at 8–10 h (2.5-fold) after heat shock. Thus, the increased accumulation of c-Fos and c-Jun RNA and immunoreactive protein following heat shock temporally parallels an increase in AP-1 DNA-
binding activity.

EMSA Supershift Assay of Heat-induced AP-1 DNA binding Using Anti-Fos and Anti-Jun—To determine whether heat-induced activation of AP-1 DNA binding results from an increase in the DNA binding of complexes, which contain c-Fos and/or c-Jun, supershift experiments were performed using cell extracts from HeLa cells 8 h following heat shock (Fig. 4, lane 1). Anti-Fos (Fig. 4, lane 2), anti-Jun antibody (Fig. 4, lane 3), or cold competitor DNA containing the AP-1 consensus motif (Fig. 4, lane 4) were added to cell extracts 10 min prior to addition of radiolabeled AP-1 oligomer. Fig. 4, lanes 2 and 3 demonstrate that the activated AP-1 complexes from heated cells contain immunoreactive c-Fos and c-Jun proteins. These results indicate that c-Fos and c-Jun proteins are present in the supershifted AP-1 complexes and suggest that the accumulation of c-Fos and c-Jun proteins (seen in Fig. 2) in response to heat contributes to the formation of heat-induced activated AP-1 complexes.

Immunodepletion of Ref-1 Impairs Inducible, but Not Basal, AP-1 DNA-binding Activity—Because AP-1 DNA-binding activity is increased by heat and Ref-1 is known to regulate AP-1 activity through a redox-sensitive mechanism (35, 36, 38), we next sought to assess whether Ref-1 is involved with the regulation of heat-induced increases in AP-1 DNA binding. These experiments were accomplished using a Ref-1 antibody to remove Ref-1 protein from the extracts that were used to determine the increase in AP-1 DNA-binding activity in response to heat. Briefly, NIH 3T3 and HeLa cells extracts from the experiments shown in Fig. 3 were treated with the addition of protein A and anti-Ref-1 antibody, followed by gentle shaking at 4 °C for 2 h. Cell extracts were spun at 12,000 rpm for 1 min to pellet out the Ref-1-antibody complex. 10 μg of Ref-1-immunodepleted whole cell extracts were analyzed by EMSA as described previously. Minus signs indicate Ref-1-immunodepleted extracts, and plus signs indicate that Ref-1 was not depleted. C, control.

AP-1 DNA binding in unheated NIH 3T3 (Fig. 5A, lane 1) and HeLa cells (Fig. 5B, lane 1) with Ref-1 present (immunoprecipitation was performed with protein A only) are shown as controls. The same extracts were immunodepleted of Ref-1 (Fig. 5, A and B, lanes 3), and when compared with the DNA binding in lane 1, no difference is observed. These results suggest that the presence of Ref-1 in the extracts was not required for AP-1 DNA-binding activity in the unstressed condition. As a positive control, the extracts from heated cells harvested at 8 h were immunoprecipitated with protein A only (Fig. 5, A and B, lanes 2). Similar to the results in Fig. 3, lanes 5 and 6, these positive controls demonstrated a 2.5-fold increase in DNA-binding activity (Fig. 5, A and B, lanes 2). In the Ref-1-depleted extracts from heated cells isolated at 2, 4, 6, 8, 10, and 24 h, there was no increase in AP-1 DNA-binding activity, relative to the unheated extracts (Fig. 5, A and B, lanes 4–9). These results indicate that the presence of Ref-1 in the extracts from heated cells was required for the heat-inducible increase in AP-1 DNA-binding activity.  

Restoration of Heat-induced AP-1 DNA-binding Activity following Addition of Ref-1 Immunoprecipitates to Immunodepleted Extracts—To confirm that Ref-1 from heated cells must be present in the NIH 3T3 cell extracts to observe heat-induced increases in AP-1 DNA binding, Ref-1-containing immunoprecipitates from heated and unheated cells were added back to the Ref-1-immunodepleted extracts from heated cells harvested 8 h following heat shock (Fig. 6A). When Ref-1 immunoprecipitate from heated cells was added back to Ref-1 minus extracts from heated cells (Fig. 6, lane 3), the heat-induced increase in AP-1 DNA binding was restored (compare lane 3 to lane 2). When Ref-1 immunoprecipitate from unheated cells was added to Ref-1 minus heated cell extracts (lane 4), no increase in AP-1 DNA binding was observed (compare lane 4 to lane 2). Interestingly, when Ref-1 immunoprecipitate from heated cells (8 h) was added to Ref-1 minus extracts from non-heated cells (lane 5), AP-1 DNA binding also increased (compare lane 5 to lane 1). Equal amounts of immunoprecipitated Ref-1 from control and heated cells were confirmed by Western analysis to rule out the possibility that the induction of AP-1 DNA binding was because of increased Ref-1 immuno-
times to remove any free chemical from the reaction and subsequently treated with DTT. Treatment with DTT after exposure to diamide completely restored the ability of Ref-1 from heated cells to induce AP-1 DNA binding (lanes 1 versus 5). These results suggest that alterations in the oxidation/reduction (redox) status of the Ref-1 protein from heated cells play a central role in the enhancement of AP-1 DNA-binding activity following heat shock.

**DISCUSSION**

The heat shock response is a rapid, complex, highly regulated process that involves coordinate control of multiple signal transduction pathways. The specific signaling pathways activated by elevated temperature, which result in the development of the heat shock response, are not completely understood. Interestingly, several components of the signaling pathways leading to the heat shock response are also stimulated by environmental stresses other than elevated temperature. These include exposure to hypoxia, heavy metals, amino acid analogues, and viral infections (3, 5, 43), all of which appear to cause perturbations in cellular redox status. These results have been interpreted to suggest that heat, perhaps via alterations in cellular metabolism, produces a condition of oxidative stress similar to that seen with other forms of cellular stress (10–17). If this were true, it might be expected that cellular pathways that sense changes in intracellular redox might also respond to heat shock. Therefore, given that several early response genes appear to play a role in the cellular response to oxidative stress, we questioned whether these same signal transduction pathways could be activated in the cellular response to heat. The central findings of our work show that heat shock leads to the induction of the early response genes, c-Fos and c-Jun, as well as increasing AP-1 DNA-binding activity by a mechanism that appears to involve the redox-sensitive signaling protein, Ref-1.

To investigate the role of early response genes in cellular responses to heat shock, NIH 3T3 and HeLa cells, representing immortalized and malignant cell lines, respectively, were utilized. These experiments demonstrate: 1) increased accumulation of c-Fos and c-Jun mRNA, protein, and AP-1 DNA-binding activity in response to heating in a temporally consistent manner; 2) that the induction of AP-1 DNA-binding activity is dependent upon Ref-1; 3) that Ref-1 appears to regulate heat-inducible, but not basal, AP-1 DNA-binding activity; and 4) that Ref-1 signaling in response to heat is redox-regulated. Interestingly, the kinetics of induction of these changes in early response genes were somewhat delayed following heat shock (2–10 h) as compared with other forms of environmental stress that perturb cellular redox more immediately, such as phorbol esters and ionizing radiation. This may reflect an inherent difference in the mechanism by which heat perturbs intracellular oxidation/reduction reactions as compared with other agents.

**Immunodepletion experiments and re-addition of immunoprecipitated Ref-1 to immunodepleted EMSA cell extracts demonstrated that Ref-1 from heated cells must be physically present in the binding reaction for the heat-induced induction of AP-1 DNA binding to be observed. Interestingly, when immunoprecipitated Ref-1 from heated cells harvested at 8 h was added to Ref-1-immunodepleted EMSA cell extracts from non-heated cells, an increase in AP-1 DNA binding was observed. These results suggest that Ref-1 is altered in a specific way following heat such that it participates in the activation of AP-1 DNA binding even in extracts from non-heated cells. Furthermore, these results support the conclusion that Ref-1 from heated cells must be physically present in the EMSA reactions to pass a "signal" to the AP-1 transcription factor.
Ref-1 is a bifunctional protein that acts as a DNA repair enzyme as well as a stimulator of DNA binding of transcriptional factors by a redox-dependent mechanism (35, 36, 40). Ref-1 protein purified from HeLa cells increases the DNA-binding activity of c-Fos and c-Jun through critical cysteine residues (Fos Cys-154 and Jun Cys-272) shown to be sensitive through the Ref-1 protein to activate the DNA binding capability of the AP-1 complex.

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ACKNOWLEDGMENTS—We thank Drs. Joseph L. Roti Roti and Andrei Laszlo for critical comments on the manuscript. We also thank Carla Thuman and Kathy Bles for assistance with preparation of this manuscript.

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