Influence of Aging and Caloric Restriction on Activation of Ras/MAPK, Calcineurin, and CaMK-IV Activities in Rat T Cells (44475)

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Abstract. **The signaling cascade mediated by Ras ($p21^{ras}$) and MAPK (mitogen-activated protein kinase) and calcium/calmodulin regulating enzymes, calcineurin (CaN) and CaMK-IV, are considered to be essential for T-cell growth and function. In the present study, the effect of aging and caloric restriction (CR) on the induction of Ras and MAPK activation by concanavalin A (ConA) was studied. Splenic T cells were isolated from young (4–6 months) and old (22–24 months) rats that had free access to food (control group), and from caloric restricted old (22–24 months) rats that beginning at 6 weeks of age were fed 60%(40% caloric restriction) of the diet consumed by the control rats. We found that the induction of Ras activity in T cells isolated from control old rats was lower ($P < 0.001$) than that in control young rats. However, the levels of Ras activity in T cells isolated from CR old rats were similar to the levels in the age-matched control rats. The induction of MAPK activity in T cells isolated from control old rats and CR old rats was significantly less than in T cells isolated from control young rats, and caloric restriction significantly ($P < 0.05$) reduced the age-related decline in MAPK activation. We also measured the induction of CaN and CaMK-IV activities by ConA in T cells from control young and old and CR old rats. The induction of both CaN and CaMK-IV activity decreased with age. Caloric restriction significantly ($P < 0.05$) reduced the age-related decline in CaN activity, but had no significant effect on CaMK-IV activity. The changes in Ras/MAPK activation and in CaN and CaMK-IV activity with age or with CR were not associated with alterations in their corresponding protein levels. Thus, caloric restriction has a differential effect on the activation of the upstream signaling molecules that are altered with age.**
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Caloric restriction (CR) has been the subject of intensive research and is known to be the most efficacious means of increasing longevity and reducing pathology in laboratory rodents (1–3). In addition, CR has been found to influence a wide variety of age-sensitive immunologic parameters such as mitogen-induced lymphocyte proliferation and IL-2 gene expression. Overall, the immunologic status of rodents fed a CR diet is superior to the immunologic status of nonrestricted animals (4). In an earlier study, we showed that the induction of IL-2 expression (activity and mRNA level) decreased with age, and CR partially reversed the age-related decline in IL-2 expression (5). In a subsequent study, we found that the increase in IL-2 expression with CR was correlated with an increase in the binding activity of the transcription factor NFAT, which plays a major role in IL-2 transcription (6). This increase in NFAT binding activity with CR was associated with an increase in the expression of c-*fos*, which is a component of the NFAT protein complex (6). Although it has been demonstrated that CR augments the immunologic function, it is

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currently unclear at the biochemical and molecular level how CR enhances T-cell function.

The events that are initiated by antigenic or mitogenic stimuli trigger a number of signal transduction pathways that culminate in the expression of cytokine genes and proliferation. The early signaling event most proximal to the T-cell receptor (TCR)/CD3 complex is the activation of *src* family of protein tyrosine kinases (PTKs), (i.e., Lck and Fyn and *syc* family of PTKs (ZAP-70). Activation of PTKs is coupled to the stimulation of an inositol lipid specific, phospholipase C-γ (PLCγ1) (7–9). This enables the TCR activation complex to regulate the mobilization of calcium through the activation of the inositol 1,4,5-triphosphate (IP3) pathway and activation of protein kinase C (PKC) through the diacylglycerol pathway (10, 11). Another PTK-mediated signaling event that originates from TCR involves the guanine nucleotide binding protein Ras ($p21^{ras}$) (12, 13). The $p21^{ras}$ protein plays a crucial role as a molecular switch, controlling diverse processes including cytokine gene expression and proliferation. Transmission of the stimulatory signals from TCR to the nuclear target appear to involve the regulation of the activity of a family of kinases known as MAPKs (mitogen-activated protein kinases) or ERKs (extracellular regulated kinases (14, 15). T cells express at least two isoforms of MAPK:ERK1 ($p44^{MAPK}$) and ERK2 ($p42^{MAPK}$) that are activated in response to stimulation (16). Once they become activated, they translocate to the nucleus where they regulate the phosphorylation of transcription factors that are involved in the transcriptional activities of immediate-early genes such as c-*myc*, c-*fos*, and c-*jun* (17, 18).

Research over the past 5 years has shown that the calcium/calmodulin-dependent phosphatase, calcineurin (type 2B serine/threonine phosphatase), plays a crucial role in calcium-mediated signaling events in T cells (19–21). Calcineurin (CaN) is essential for the regulation of the transcription factor NFAT, which plays a predominant role in IL-2 transcription. CaN is involved in transactivation (dephosphorylation and nuclear translocation) of the cytoplasmic components of the NFAT protein complex, and its inhibition by immunosuppressive drugs such as cyclosporine A and FK506 leads to the suppression of lymphokine gene expression (21). In addition to CaN, other calcium/calmodulin-binding enzymes, such as the multifunctional calcium/calmodulin-dependent protein kinase (CaMK) type IV/Gr (CaMK-IV), play an important role in the regulation of calcium in various cells including T cells (22–24). CaMK-IV phosphorylates the nuclear protein CREB (cAMP-response element binding protein) on a serine (Ser^{133}) residue, and it appears to contribute to increased transcription of immediate early genes containing CRE regulatory sequences (25, 26).

Several laboratories including our laboratory have studied the effect of aging on early signal transduction events that occur after stimulation of T cells. For example, we have recently reported that the induction of MAPK activity, but not protein levels, in T cells from rats decreased with age, and this decline was paralleled by a decline in $p21^{ras}$ activity (27). Because caloric restriction attenuates the age-related changes in the expression of a variety of genes, including IL-2 (4, 28), we investigated the effect of CR and aging on the induction of Ras and MAPK activation. In addition, we also studied the influence of CR and aging on the induction of the calcium regulating enzymes, CaN and CaMK-IV, in T cells from rats. Our study showed that the induction of Ras and MAPK activation and CaN and CaMK-IV activities by ConA decreases with age, and that CR attenuates the age-related decrease in MAPK and CaN activity; however, it has no significant effect on Ras or CaMK-IV activity.

Materials and Methods

Animals and Diets. Male Fischer 344 rats (specific pathogen-free) were obtained at 3 weeks of age from Harlan Sprague-Dawley (Indianapolis, IN). All procedures for handling the rats were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio, and the subcommittee for Animal Studies at the Audie L. Murphy Memorial Veterans Hospital. The rats were caged individually in a barrier facility with a 12-hr light/dark cycle and were fed a semisynthetic soy protein diet (5770 *M*-S Vitamin Fortified RP 101 Purified Diet; Purina Mills, Inc., St. Louis, MO), which consisted of the following ingredients: 21% RP101 soy protein isolate, 15% sucrose, 45.99% dextran, 6% corn oil, 5% Ralston Purina mineral mix, 3% solka floc, 0.35% DL-Methionine, 0.33% choline chloride, and 2% Ralston Purina vitamin mix. The rats had free access to water (HCl was added to water for clarity and the pH was maintained at 3.1), as previously described (29). At 6 weeks of age, the rats were randomly assigned to two groups. The control group was given free access to the diet, and the restricted group received 60% of the diet consumed by the control rats as described by Yu *et al.* (30). The soy protein diet prevents the progression of chronic nephropathy, which has been reported in this strain of rats fed diets containing other proteins (31). The median survival of the restricted rats was 938 days compared to a median survival of 813 days for the control rats that had free access to the diet. Three groups of rats were used in these experiments: Young (4–6 months), old (22–24 months) rats that had free access to food (control), and caloric restricted old (22–24 months) rats.

Isolation of Lymphocytes. Rats were decapitated, and spleens were removed aseptically. Single-cell suspensions were obtained, and erythrocytes were removed using Lympholyte-R (Accurate Chemical and Scientific Corporation, Westbury, NY). B cells and macrophages were removed from T cells by a nylon wool column and the panning technique as previously described (32). The purity of the T-cell population obtained by the panning technique is generally between 90%–95% as determined by flow cytometry. T cells were resuspended in RPMI-1640 medium,

which was supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were cultured in the presence or absence of ConA (5 μg/ml), and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Immunoprecipitation and Western Blotting. Purified T cells were cultured with or without ConA for 5–15 min. Cells were lyzed in a protein lysis buffer (1% Nonidet P-40, 150 m*M* NaCl, and 20 m*M* Tris, pH 7.4) containing protease inhibitors (50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin A, and 1 m*M* polymethylsulfonyl fluroide) and then incubated on ice for 30 min. The lysates were clarified by centrifugation for 30 min at 10,000*g* at 4°C, and the supernatant was assayed for protein concentration using the Bio-Rad Protein Assay Kit. The Ras ($p21^{ras}$), MAPKs ($p44^{MAPK}$ and $p42^{MAPK}$), or CaMK-IV were immunoprecipitated from cell extracts using specific antibodies against each protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as previously described (33). Briefly, the protein lysates were incubated on ice overnight with each specific antibody followed by the addition of protein A-Sepharose (Pharmacia, Piscataway, NJ). The immune complex was collected by centrifugation at 4°C and was subsequently washed three times with lysis buffer. The kinase activity associated with each immunoprecipitated protein was measured using the kinase assay (see below). For Western blotting, protein extracts (30 μg) were boiled for 5 min with an equal volume of 2x SDS-PAGE sample buffer, and resolved on 10% or 12% SDS-PAGE. Protein from the gels were electroblotted onto nitrocellulose filters, and the filters were blocked by incubating for 2 hr at room temperature with 5% nonfat milk in PBS, 0.1% Tween-20. The filters were incubated overnight with anti-Ras, antip44 and p42 MAPK, anti-CaN or anti-CaMK-IV (Transduction Laboratories, Lexington, KY) antibody and washed three times (10 min each) with 0.1% Tween 20 in PBS. The filters were then incubated with peroxidase-labeled IgG $F(ab')_2$ as the secondary antibody and were developed using an enhanced chemiluminescence detection system, ECL (Amersham, Arlington Heights, IL).

Assay for $p21^{ras}$ Activity. Analysis of $p21^{ras}$-bound GTP/GDP was performed as described by Downward *et al.* (12). Briefly, 100 million cells were labeled with 0.5 mCi of (γ-^{32}P)ortho-phosphate (Dupont New England Nuclear, Boston, MA) for 2 hr in phosphate-free RPMI-1640 medium with 20 m*M* Hepes, pH 7.2. Cells were then stimulated with ConA for 10 min, and the activity of the immunoprecipitated $p21^{ras}$ was assessed by measuring the percentage of bound GTP. The $p21^{ras}$-bound guanine nucleotides (bound GTP and GDP) were eluted with 0.5 m*M* GTP, 0.5 m*M* GDT, 2 m*M* EDTA, 2 m*M* DTT, and 0.2% SDS for 30 min at 68°C. The supernatants were lyophilized, resuspended in 15 μl of 50% ethanol/water, and applied to a polyethyleneimine-cellulose thin-layer chromatography plate. Nucleotides were resolved in 1 *M* of KH_2PO_4, and the plates were exposed to X-OMAT AR Kodak film for 24 hr at –70°C. Spots corresponding to GTP and GDP were identified and excised, and the amount of radioactivity was counted by liquid scintillation as described by Tridandapani *et al.* (34).

Assay for MAPK Activities. The MAPK activity was measured using the method described by Liu *et al.* (35). Briefly, T cells were cultured in the presence or absence of ConA for 15 min, and the activities of the immunoprecipitated p44 and p42 MAPK were measured. The immunoprecipitated proteins (25 μl) were added to 25 μl of MAPK phosphorylation buffer (20 m*M* $MgCl_2$, 3 m*M* $MnCl_2$, 3 m*M* 2-ME, 50 μ*M* ATP, 3 μCi (γ-^{32}P) ATP/tube), plus 1 mg/ml of synthetic MAPK substrate peptide (APRTPGGRR), corresponding to amino acids 95–98 of bovine myelin basic protein (Upstate Biotechnology Inc., Lake Placid, NY), which is uniquely phosphorylated by MAPK (36, 37). The mixture was incubated for 20 min at 30°C, and then spotted on P81 phosphocellulose paper disks (Whatman, Hillsboro, OR). The disks were washed several times in 0.85% phosphoric acid and once in acetone, dried and counted in a scintillation counter for ^{32}P incorporation into the substrate peptide 37).

Assay for Calcineurin and CaMK-IV Activity. T cells were cultured in the presence or absence of ConA for 5 min. Cells were lyzed in a hypotonic lysis buffer consisting of 50 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.1 m*M* EGTA, 0.5 m*M* DTT, 50 μg/ml PMSF, 50 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cells were lyzed by freezing and thawing, and a protein assay was performed on cell lysates using protein assay kit (Bio-Rad). Calcineurin phosphatase activity was measured as described by Fruman *et al.* (38). Briefly, the 19-amino-acid synthetic peptide substrate [Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-Glu], corresponding to a sequence in the RII subunit of cAMP-dependent kinase, was synthesized by standard procedures (Molecular Biology Core Facility, University of Texas Health Science Center, San Antonio, TX). Phosphorylation of the serine residues with [γ-^{32}P]-ATP was performed as described (38) with the catalytic subunit of cAMP-dependent protein kinase (Sigma Chemical Co., St. Louis, MO). Phosphatase assay was performed using lysates from the resting or ConA-stimulated T cells isolated from the animals. Reaction mixtures contained 50 μg of protein lysate from ConA-stimulated T cells or hypotonic lysis buffer (control), 10 μ*M* ^{32}P-labeled peptide substrate, 20 m*M* Tris pH 8, 100 m*M* NaCl, 6 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 0.1 mg/ml of bovine serum albumin, and 0.1 m*M* $CaCl_2$. After 15 min incubation at 30°C, reactions were terminated by the addition of 0.5 ml of 100 m*M* potassium phosphate buffer pH 7.0 containing 5% trichloroacetic acid. Free inorganic phosphate was isolated by Dowex cation-exchange (Bio-Rad) chromatography, and quantitated by scintillation counting as described. The number of picomoles of phosphate released was calculated by using the specific activity of the substrate measured on the day of the assay. Specific activity

was determined by measuring the cpm in 20 μl of 300 pmol ^{32}P-labeled phosphopeptide as described by Fruman *et al.* (38). Analysis of CaMK-IV activity was performed according to the method described by Park and Soderling (39) using syntide-2 peptide substrate. The reaction mixture contained immunoprecipitated CaMK-IV protein from the unstimulated or ConA-stimulated cells, 50 m*M* HEPES, pH 7.5, 2 m*M* DTT, 40 μ*M* of peptide substrate (syntide-2), 0.2 m*M* [γ-^{32}p]-ATP, 10 m*M* magnesium acetate, 5 μ*M* PKI, and 2 μ*M* PKC inhibitor peptides. The reaction proceeded for 10 min at 30°C and was then terminated by spotting 10 μl aliquots onto phosphocellulose P81 filters. The filters were rinsed five times with 0.75% phosphoric acid, then washed in acetone for 2 min. The bound radioactivity was quantitated with a scintillation counter, and CaMK-IV activity was expressed as pmol/min/mg protein.

Statistical Analysis. The Mann–Whitney *U*-test was used to make comparisons between the two independent samples, control young and old rats, or control young rats and calorie-restricted old rats. The Wilcoxon Signed Rank Test was used to determine the significant differences between the age-matched groups (i.e., control old rats versus calorie-restricted old rats) (40).

Results

Effect of Caloric Restriction and Aging on Ras/MAPK Activation. In our previous study, we found that ConA rapidly induced the activation of Ras and MAPK in T cells from rats, and the maximum activity was observed at 10 min and 15 min, respectively, after stimulation (27). To determine the influence of CR and aging on Ras activity, T cells from control young and old rats or CR old rats were incubated with or without ConA for 10 min or 15 min, and the activity associated with immunoprecipitated p21^{ras} or p44 and p42 MAPK was measured. Ras activation was assessed by measuring the accumulation of GTP- and GDP-bound p21^{ras} protein, and MAPK activity was measured by the phosphorylation of a synthetic peptide substrate composed of residues of 95–98 of the bovine myelin basic protein. Figure 1 shows data on the effect of aging and caloric restriction on Ras and MAPK activation. The basal levels of Ras activity were similar in T cells from control young and old rats and CR old rats. ConA-stimulation of T cells from young rats resulted in an increase of 520% in Ras activity, whereas the Ras activity increased ≈ 230% and 280% in T cells from control old rats and CR old rats, respectively. The induction of Ras activity by ConA was slightly higher in T cells isolated from CR old rats than the age-matched control rats; however, this difference was not statistically significant.

The data in Figure 1 also show that ConA-stimulation of T cells from control young rats resulted in an over 400% increase in MAPK activity. Whereas MAPK activity increased ≈ 140% and 270% in T cells from control old rats and CR old rats, respectively. Thus, the induction of MAPK activity was 36% higher ($P < 0.05$) in T cells isolated from CR old rats than the age-matched control. We also measured the effect of age and CR on the induction of JNK activation and found that the induction of JNK activity did not change significantly with age or with CR (data not shown). The changes in Ras/MAPK activity with age and with CR were not associated with changes in protein levels because Western blot analysis showed that the levels of Ras (p21^{ras}) and MAPK (p44 and p42 proteins) were similar in T cells from control young and old rats and CR old rats (data not shown).

Effect of Caloric Restriction and Aging on Calcineurin and CaMK-IV Activation. Recent studies suggest that calcium regulating enzymes (i.e, CaN and CaMK-IV) are important in T-cell signaling and IL-2 gene expression (20–24). Using a synthetic peptide corresponding to the phosphorylation site of the RII subunit of cAMP-dependent protein kinase (38), the CaN phosphatase activity was measured in protein extracts from unstimulated and ConA-stimulated T cells isolated from rats. Figure 2 shows that the basal levels of CaN were similar in T cells from control

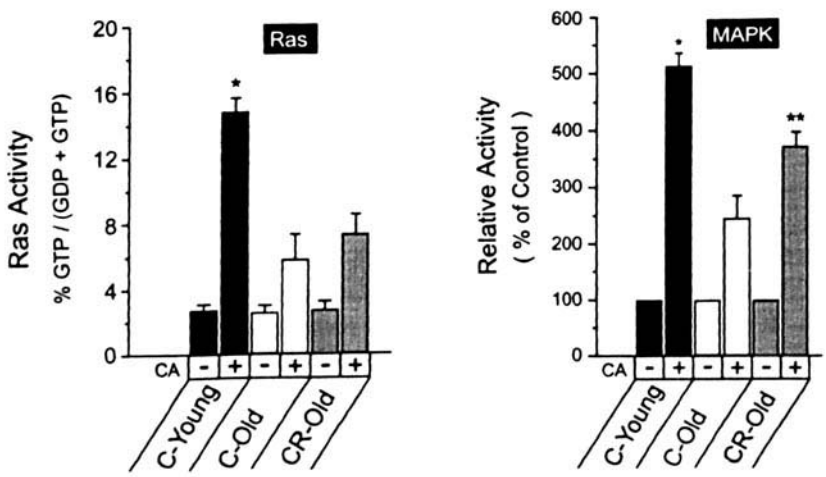

Figure 1. Effect of age and caloric restriction on the induction of Ras and MAPK activation in T cells from rats. Splenic T cells were isolated from control (C) young and old rats or calorie-restricted (CR) old rats. Cells were incubated with or without ConA for 10 min to measure Ras activity (left) or for 15 min to measure MAPK activity (right) as described in Materials and Methods. The Ras activity is expressed as the percentage ratio of GTP-p21^{ras} over total (GDP-p21^{ras} plus GTP-p21^{ras}). The data for MAPK activity are expressed as percentage activity in ConA (CA)-stimulated cells over the unstimulated cells. Each point represents the mean ± SD for data obtained from three experiments (for the Ras activity) or from four experiments (for the MAPK activity), and each experiment was pooled from two rats. *The value for young rats was significantly different from the values for the control old rats at the $P < 0.001$. **The value for caloric restricted old rats was significantly different from the value for the age-matched control at the $P < 0.05$.

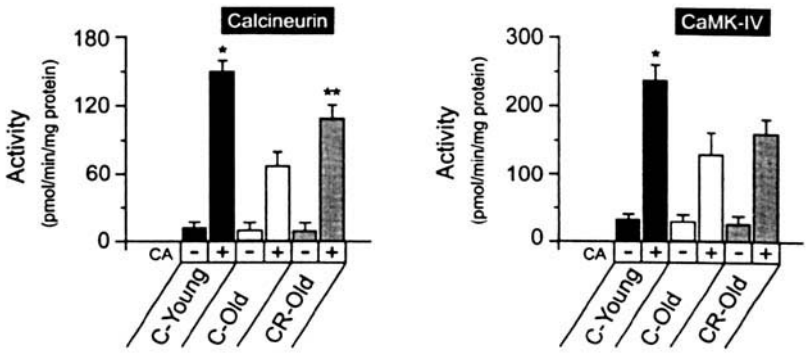

Figure 2. Effect of age and CR on the induction of calcineurin (CaN) and CaMK-IV activities in T cells from rats. Splenic T cells were isolated from control (C) young and old rats or caloric restricted (CR) old rats. Cells were cultured in the presence or absence of ConA. After 5–10 min of incubation, cells were lyzed and the protein extracts were assayed for CaN phosphatase activity and CaMK-IV activity as described in the Methods. Each point represents the mean ± SD for data obtained from four experiments for the CaN assay, and three experiments for the CaMK-IV assay, each experiment was pooled from two rats. * The value for young rats was significantly different from the values for the control old rats and calorie-restricted old rats at $P < 0.05$. **The value for caloric restricted old rats was significantly different from the value for the age-matched control at $P < 0.05$.

young and old rats and CR old rats. ConA stimulation resulted in a marked increase in the rate of dephosphorylation of the synthetic peptide substrate in T cells from young and old rats. CaN phosphatase activity was ≈ 57% and 32% lower in T cells isolated from control old rats and CR old rats, respectively, than in those from control young rats. The induction of CaN phosphatase activity in T cells isolated from CR old rats was 38% higher ($P < 0.05$) than that of the age-matched counterpart. Figure 2 also shows data in which we measured the induction of CaMK-IV activity using syntide-2 peptide as a substrate. The rate of ^{32}P incorporation by the peptide substrate after ConA was ≈ 46% and 37% lower in T cells isolated from control old rats and CR old rats, respectively, compared with the control young rats. Although the induction of CaMK-IV activity was slightly higher in T cells from CR old rats than control old rats, this difference was not statistically significant. Thus, our data show that the activation of CaN phosphatase activity and CaMK-IV activity decreased with age and that CR attenuated the age-related decrease in CaN activity, but not CaMK-IV activity.

Figure 3 shows the levels of CaN and CaMK-IV protein measured by Western blot analysis using antibodies against CaN (p61) and CaMK-IV proteins. The levels of CaN and CaMK-IV proteins were similar in ConA-stimulated T cells from control young and old rats and CR old rats. Thus, the changes in CaN and CaMK-IV activity with age or with CR were not due to changes at the protein level.

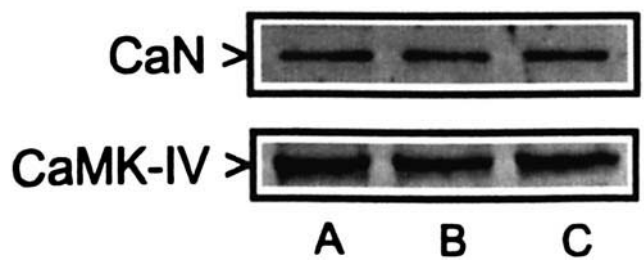

Figure 3. Effect of age and CR on the protein levels of CaN and CaMK-IV in T cells from rats. Protein lysates from ConA-stimulated cells from (A) control young and (B) old or (C) CR old were subjected to SDS-PAGE. Following electrotransfer, the blots were probed with anti-calcineurin (p61) or anti-CaMK-IV antibody, and the immunoreactive protein band corresponding to calcineurin and CaMK-IV was visualized by ECL. Data were obtained from pooled samples of three spleens from each group. Similar results were obtained from the two experiments.

Discussion

T cell activation is initiated when an antigenic peptide is recognized by the antigen receptor of the T cell. This recognition event promotes sequential activation of a network of signaling molecules such as kinases, phosphatases, and adaptor proteins that couple the stimulatory signal received from TCR to intracellular signaling pathways (7, 10). The coordinated activation of these signaling molecules is sufficient to stimulate the activation of transcription factors and the expression of immediate-early genes that are crucial in the regulation of T cell function. Because T cell responses such as gene expression, proliferation, and differentiation are critically dependent on signal transduction cascades, several studies have focused on the effect of age on the activation or the levels of signal transduction molecules. For example, our laboratory has recently shown that the induction of Ras and MAPK activity by ConA decreased with age, and that this decrease was paralleled by a decrease in the induction of TCR-associated protein tyrosine kinases, Lck ($p56^{lck}$) and ZAP-70 (27). Other laboratories have also reported that the induction of MAPK and MEK is reduced with increasing age in T cells from mice and humans (41). Thus, the available data indicate that the induction of MAPK activity in T cells decreases with age.

Although it has been demonstrated that the activation of MAPK decreases with age, there is essentially no information on the influence of CR on the induction of the upstream signaling molecules (e.g., Ras or MAPK activity). Therefore, the basic objective of the present study was to determine if CR altered the age-related decline in MAPK activity and if the changes were correlated with changes in Ras activation. The present study showed that the induction of Ras activity decreases significantly with age, but CR had no significant effect on the age-related decrease in Ras activation. Because the activation of Ras is coupled with the ac-

tivation of downstream signaling molecules (i.e., MAPK) and because the activation of MAPK decreases with age (41), we then studied whether CR alters the age-related decrease in MAPK activity in T cells. The results showed that the induction of MAPK activity was significantly less in T cells from control old and CR old rats than T cells from control young rats (Fig. 1). More importantly, the present study showed that 40% caloric restriction partially reversed the age-related decline in MAPK activation.

Activation of T cells results in a transient increase in intracellular free calcium ion concentrations, which leads to the activation of calcium/calmodulin-dependent enzymes such as calcineurin (CaN) and the multifunctional CaMK-II and CaMK-IV/Gr. Over the past several years, it has been shown that the calcium/calmodulin-dependent phosphatase calcineurin is crucial for the regulation of the transcription factor NFAT that is involved in IL-2 transcription (19, 20). In response to an increase in the intracellular levels of calcium, calcineurin is activated, which dephosphorylates the cytoplasmic component (NFAT-c) of the NFAT protein complex. The dephosphorylated form of NFAT-c translocates into the nucleus and forms a complex with the nuclear components (*fos*/*jun*-*elf*-1) of NFAT resulting in the stimulation of IL-2 transcription (19). In addition, recent studies have demonstrated that the calcium/calmodulin-dependent kinase type IV/Gr (CaMK-IV) plays an important role in the upregulation of the transcriptional activity of the c-*fos* promoter through phosphorylation of the CREB and serum response factor (SRF) (42, 43). Therefore, the second objective of the present study was to examine whether the activation or the level of these calcium regulating enzymes (CaN and CaMK-IV) is altered with age and whether these changes are affected by CR. We found that the induction of CaN phosphatase activity and CaMK-IV kinase activity by ConA, decreased with age and caloric restriction, partially reversed the age-related decline in CaN activation but not CaMK-IV activity (Fig. 2). The decrease in CaN and CaMK-IV activity with age or with CR was not due to changes in their protein levels (Fig. 3).

This report is the first study on the effect of caloric restriction and aging on the induction of Ras and MAPK activation and CaN and CaMK-IV activities in T cells. Based on the current model of signal transduction events, the increase in MAPK activation with CR could occur at least by two distinct mechanisms. First, the increase in the MAPK activity with CR could arise from increased activity of the proximal signaling molecules such as MEK. In other words, more MAPK activity is observed in T cells of CR old rats because more MEK activity is present in these cells. Second, the increase in activity of MAPK may be due to the downregulation of MAPK phosphatase (MPK-1), which plays a role in the regulation of MAPK activity. That is, similar levels of MAPK protein are present in T cells from control old and CR old rats; but in response to stimulation, the activity of MPK-1 that is involved in dephosphorylation and downregulation of MAPK, decreases in the T cells from CR old rats. Our data show that the influence of CR on signal transduction events can vary considerably from one signaling molecule to another. For example, CR partially reverses the age-related decline in MAPK and CaN activities, but it appears to have no effect in Ras or CaMK-IV activation. At the present time, it is not known why CR reduces the age-related decrease in MAPK and CaN activities, but not Ras or CaMK-IV activity. Thus, it would be of interest in the future to determine the mechanism by which CR alters the activity of one group of signaling molecules and not others.

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