Curcumin-induced inhibition of cellular reactive oxygen species generation: Novel therapeutic implications

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There is evidence for increased levels of circulating reactive oxygen species (ROS) in diabetics, as indirectly inferred by the findings of increased lipid peroxidation and decreased antioxidant status. Direct measurements of intracellular generation of ROS using fluorescent dyes also demonstrate an association of oxidative stress with diabetes. Although phenolic compounds attenuate oxidative stress-related tissue damage, there are concerns over toxicity of synthetic phenolic antioxidants and this has considerably stimulated interest in investigating the role of natural phenolics in medicinal applications. Curcumin (the primary active principle in turmeric, *Curcuma longa* Linn.) has been claimed to represent a potential antioxidant and antiinflammatory agent with phytonutrient and bioprotective properties. However there are lack of molecular studies to demonstrate its cellular action and potential molecular targets. In this study the antioxidant effect of curcumin as a function of changes in cellular ROS generation was tested. Our results clearly demonstrate that curcumin abolished both phorbol-12 myristate-13 acetate (PMA) and thapsigargin-induced ROS generation in cells from control and diabetic subjects. The pattern of these ROS inhibitory effects as a function of dose-dependency suggests that curcumin mechanistically interferes with protein kinase C (PKC) and calcium regulation. Simultaneous measurements of ROS and Ca^{2+} influx suggest that a rise in cytosolic Ca^{2+} may be a trigger for increased ROS generation. We suggest that the antioxidant and antiangeogenic actions of curcumin, as a mechanism of inhibition of Ca^{2+} entry and PKC activity, should be further exploited to develop suitable and novel drugs for the treatment of diabetic retinopathy and other diabetic complications.

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1. Introduction

Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes mellitus (Mercuri *et al* 2000; Brownlee 2001; Rosen *et al* 2001; Bonnefont-Rousselot 2002; Ceriello 2003). Free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance. Therefore, oxidative stress is considered a common endpoint of chronic diseases like diabetes (Baynes 1991) and is often characterized by

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Abbreviations used: DCF, 2′,7′-dichlorofluorescein; HBS, HEPES buffered saline; IP3, inositol-triphosphate; PKC, protein kinase C; PMA, phorbol-12 myristate-13 acetate; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; Tg, thapsigargin.

an increase in the steady state concentration of reactive oxygen species (ROS), such as H_2O_2, lipid peroxides, superoxide and hypochlorous acid. Oxidative stress can modulate a wide variety of biological processes by coupling signals at the cell surface with changes in gene expression, suggesting that multiple signalling pathways are involved. Indeed, ROS may be defined as true second-messenger molecules that regulate various signal transduction cascades upstream of nuclear transcription factors, including modulation of Ca^{2+} signalling, protein kinase and protein phosphatase pathways (Sundaresan *et al* 1995; Palmer and Paulson 1997). Therefore, it is important to explore the relationship between free radicals, diabetes, and its complications, and to elucidate the mechanisms by which increased oxidative stress accelerates the development of diabetic complications. These efforts will ultimately expand and increase our treatment options.

There is a need for screening of herbal medicines with special reference to diabetes and its complications (Balasubramanyam and Mohan 2002). The significance of *Curcuma longa* Linn. (turmeric) in health and nutrition has changed considerably since the discovery of the antioxidant properties of naturally occurring phenolic compounds. The dried rhizome of *C. longa* (used as a spice, food preservative and a colouring agent) is a rich source of beneficial phenolic compounds; the curcuminoids (Srinivasan 1953). Curcuminoids are a group of phenolics present in turmeric. These curcuminoids are chemically related to its principal ingredient; the curcumin. Three main curcuminoids were isolated from turmeric: namely; curcumin (C), demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC). In this study we have tested the antioxidant effect of curcumin as a function of changes in cellular ROS generation and explored its novel mechanisms of protection against oxidative stress.

2. Materials and methods

2.1 *Study subjects*

All study subjects were recruited from M V Diabetes Specialities Centre (MVDSC) at Gopalapuram, Chennai. Additionally, healthy volunteers were also recruited for blood sampling for the standardization of various methodologies related to cellular approaches. Statistical data analysis was performed using PC-compatible SPSS package.

2.2 *Lymphocyte isolation*

Freshly collected blood was carefully layered on top of Histopaque (1077) or Lymphoprep density gradient solution and centrifuged at 1600 rpm for 30 min. The buffy coat interface that represents lymphocytes was aspirated and suspended in 1 ml HEPES buffered saline (HBS, pH 7·4) containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Curcumin, thapsigargin, dicholorofluorescein diacetate, Fura2-AM and phorbol-12 myrsitate-13 acetate (PMA) were obtained from Sigma-Aldrich Co., USA. All other reagents used were of analytical grade.

2.3 *Determination of intracellular ROS production*

To measure intracellular ROS production, cells were loaded with 10 μM dichlorofluorescein diacetate (DCFH-DA) for 45 min. ROS levels were measured (using Fluoromax-3 spectrofluorimeter with an excitation set at 485 nm and emission at 530 nm) as a change in the fluorescence because of the conversion of non-fluorescent dichlorofluorescein diacetate to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) in the cells. Aliquots of cell suspension (100 μl) were incubated for 10 min with curcumin and resuspended in HBS prior to each experiment. This potentially eliminated the interference of curcumin in fluorescence assays.

2.4 *Measurement of Ca^{2+} influx*

Lymphocyte suspension was incubated with 5 μM Fura-2 AM for a minimum of 30 min at 37°C and delivered as 100 μl aliquots. Prior to each experiment, cells were centrifuged for 5–10 s at room temperature, resuspended in 100 μl of HBS, and injected into cuvettes containing 3 ml of assay buffer. Ca^{2+} influx was measured using a methodology of Mn^{2+} quenching of intracellular fura (Balasubramanyam *et al* 1993). Here, Mn^{2+} is used as a surrogate marker of measuring Ca^{2+} entry. When measured at an isobestic wavelength of 360 nm, the Mn^{2+} quenching of intracellular fura is a relative measurement of Ca^{2+} entry into the cells.

3. Results

Curcumin-induced cellular changes were studied using two agents viz., thapsigargin (Tg) and PMA which specifically interfere with calcium and protein kinase C (PKC) metabolism, respectively, and increase cellular ROS production. Tg is a pharmacological specific inhibitor of sarcoplasmic endoplasmic reticular calcium (SERCA). ATPases and cells treated with Tg exhibit depletion of intracellular Ca^{2+} stores and subsequent rise in cytosolic Ca^{2+}, as a function of store-operated Ca^{2+} influx (Thastrup

et al 1990; Balasubramanyam *et al* 1993). PMA is a common activator of cellular PKC isozymes (Ohno and Nishizuka 2002). Figure 1 shows the Tg-induced ROS generation in cells from control and diabetic subjects, with a general trend of increased ROS in diabetic patients. In cells pretreated with curcumin (100 μM), there was a total absence of Tg-induced ROS generation. The extent of curcumin inhibition of Tg-induced ROS generation was almost same in cells from control (88% ± 4·5) and diabetic (92% ± 1·7) subjects. When lymphocytes were treated with PMA – a PKC activator – PMA induced increased levels of ROS generation (figure 2). In general, PMA-induced ROS generation was less in cells from diabetes patients compared to control subjects. However, the extent of curcumin inhibition of PMA-induced ROS generation was almost the same in cells from control (91% ± 3·6) and diabetic (88% ± 4·34) subjects. To prove that the effect of curcumin was not non-specific but rather a mechanistic biological phenomenon, dose-dependent effects of curcumin were tested on both Tg-induced and PMA-induced ROS generation. Curcumin dose-dependently (0·1 μM to 100 μM) inhibited PMA-induced cytosolic ROS levels (figure 3). Similar inhibitory effects of curcumin (0·3 μM to 100 μM) were also observed on Tg-induced ROS generation (data not shown). These results demonstrate specific biological effects of curcumin on important messenger molecules, such as cytosolic calcium and PKC.

Earlier studies indicated that Tg-induced cellular Ca^{2+} entry was higher in cells from diabetes patients (Balasubramanyam *et al* 2001), and an increase in cytosolic Ca^{2+} via store-operated Ca^{2+} entry is a prerequisite for increased intracellular ROS generation (Balasubramanyam *et al* 2002). If there is an existence of a cross-talk signalling of increased ROS and cytosolic calcium, it should be possible to demonstrate in an experiment where simultaneously ROS and Ca^{2+} entry are measured. As shown in figure 4, Mn quenching of intracellular fura is a reliable measure of Ca^{2+} entering into the cells (dotted lines) and DCF-fluorescence is a reliable measure of intracellular ROS generation, both events initiated by Tg. This methodology clearly indicates that an increase in Ca^{2+} rise in the cell is a trigger for increased ROS generation. Interestingly, curcumin inhibits both Tg-induced Ca^{2+} entry and ROS generation, suggesting that Ca^{2+} entry inhibition might be one of the mechanisms by which curcumin exerts its antioxidant effect and inhibits ROS generation.

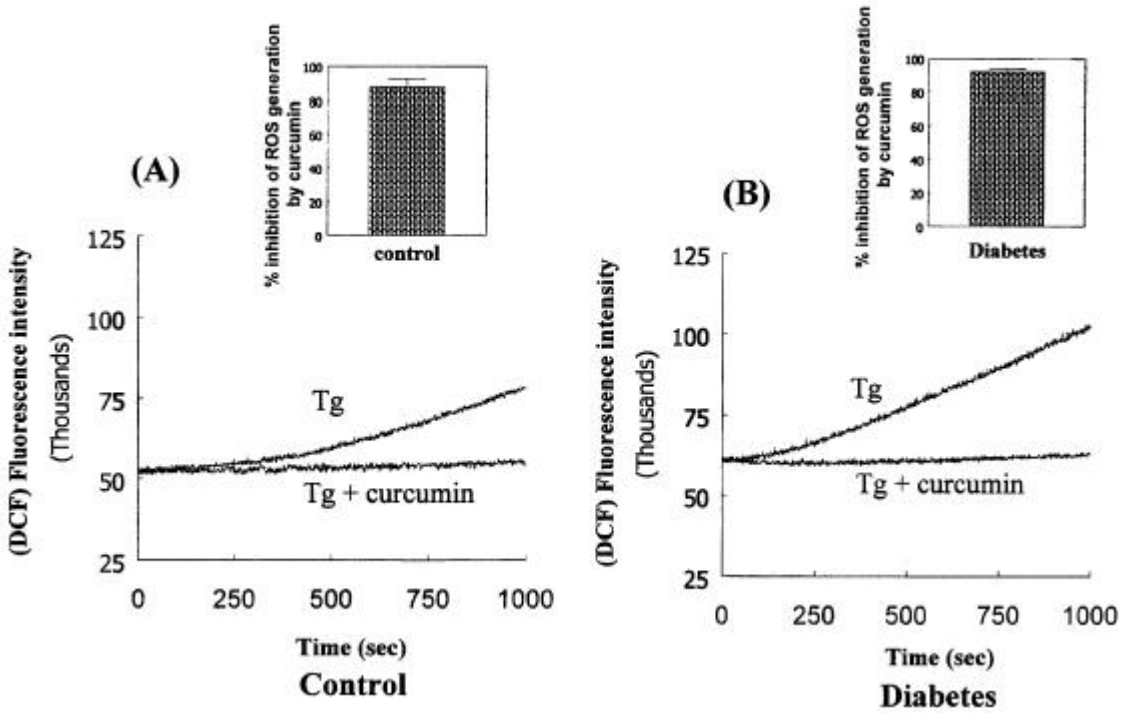

Figure 1. Curcumin inhibition of Tg-induced ROS generation in cells from control (**A**) and type 2 diabetes subjects (**B**). Freshly isolated lymphocytes were resuspended in HBS buffer and loaded with 10 μM DCF for 45 min. At time '0' thapsigargin (100 nM) was added and increase in DCF fluorescence was monitored for 1000 s, with and without curcumin (100 μM). Insets indicate percent inhibition of ROS generation by curcumin. Each bar diagram represents cumulative data obtained from 6 to 10 separate experiments.

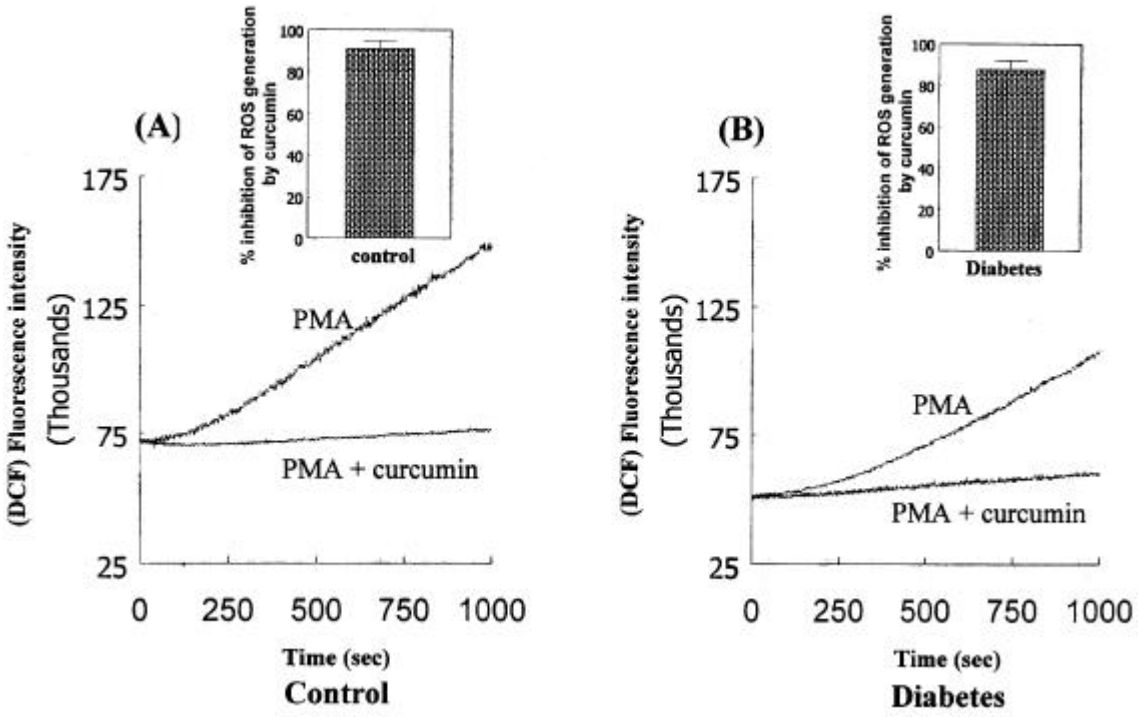

Figure 2. Curcumin inhibition of PMA-induced ROS generation in cells from control (**A**) and type 2 diabetes subjects (**B**). Experimental maneuvers were same as in figure 1.

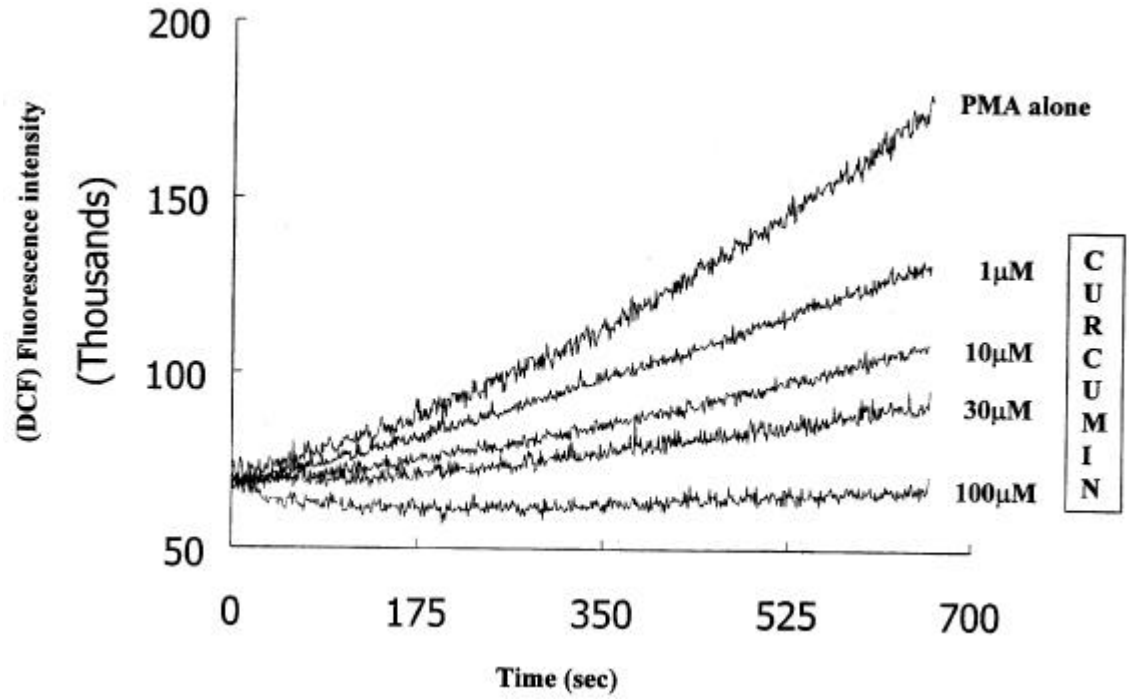

Figure 3. Curcumin dose-dependently inhibits PMA-induced intracellular ROS generation. Increases in DCF fluorescence after the addition of PMA were monitored in the absence and presence (1 μM to 100 μM) of curcumin. Representative traces of 4 separate dose-dependency experiments.

4. Discussion

Earlier studies have shown that curcumin can affect a number of cellular processes including activation of apoptosis in Jurkat T cells (Piwocka *et al* 1999), inhibition of platelet aggregation (Shah *et al* 1999; Srivastava *et al* 1995), and inhibition of inflammatory cytokine production in macrophages (Abe *et al* 1999). Curcumin has also been shown to affect the activity of a number of key enzymes such as cyclooxygenase (Zhang *et al* 1999), PKC (Liu *et al* 1993), protein tyrosine kinases (Chen and Huang 1998) and a Ca^{2+}-dependent endonuclease (Chen *et al* 1996). Many of these processes/enzymes were known to be regulated by Ca^{2+}. Cytosolic calcium is tightly controlled. The sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is one of the major mechanisms by which the low levels of cytosolic calcium (~ 100 nM) are maintained within cells. Interestingly, it has been shown that all subtypes of SERCA are inhibited to a similar degree by curcumin (Bilmen *et al* 2001). In our study, curcumin inhibits Tg-induced ROS production and Ca^{2+} entry. The likely mechanisms are: (i) curcumin apart from its effects on SERCA may also inhibit inositol-triphosphate(IP3)-sensitive Ca^{2+} channel (Dyer *et al* 2002) and thereby break the coupling of IP3-sensitive Ca^{2+} store and Ca^{2+} entry; and (ii) curcumin may inhibit cytosolic signals (enzymes or other signalling molecules) that are responsible for Ca^{2+} entry into the cells.

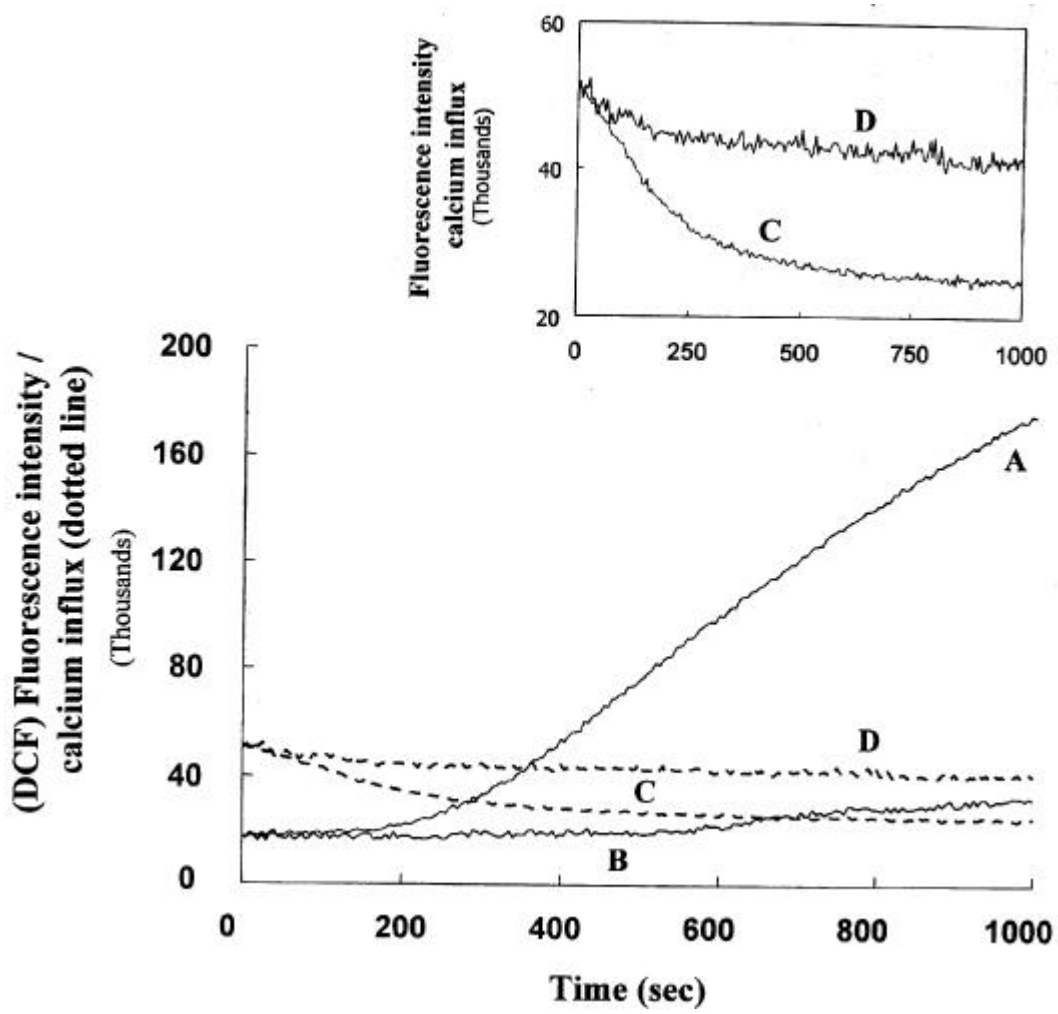

Figure 4. Methodology for simultaneous measurement of ROS generation and Ca^{2+} influx. Cells were co-loaded with both DCF and Fura and changes in respective fluorescence intensities were monitored using multiwavelength parameters in a Fluoromax-3 spectrofluorimeter. Solid lines represent increases in Tg-induced ROS generation (A) and its inhibition by curcumin (B). Dotted lines represent Tg-induced Mn^{2+} quenching (C) of intracellular fura (a surrogate measure of Ca^{2+} influx) and its inhibition by curcumin (D). Representative traces of 4 separate experiments. *Inset*: Traces (C) and (D) were separately illustrated to show the extent of Tg-induced Ca^{2+} influx and its inhibition by curcumin.

Curcumin inhibition of PMA-induced ROS generation suggests that curcumin has an inhibitory action on PKC activity. It is now recognized that low levels of oxidants can modify cell-signalling proteins and that these modifications have functional consequences (Lee *et al* 1989). For example, PKC is a logical candidate for redox modification by oxidants and antioxidants that may in part determine their cancer-promoting and anticancer activities, respectively. PKC contains unique structural features that are susceptible to oxidative modification. The N-terminal regulatory domain contains zinc-binding, cysteine-rich motifs that are readily oxidized by peroxide. When oxidized, the auto inhibitory function of the regulatory domain is compromised and, consequently, cellular PKC activity is stimulated. The C-terminal catalytic domain contains several reactive cysteines that are targets for various chemopreventive antioxidants (such as selenocompounds), polyphenolic agents (such as curcumin), and vitamin E analogues (Gopalakrishna and Jaken 2000). Modification of these cysteines decreases cellular PKC activity. Thus the two domains of PKC respond differently to two different types of agents. Oxidants selectively react with the regulatory domain, stimulate cellular PKC, and act as a signal for tumour promotion and cell growth. In contrast, antioxidant chemo preventive agents react with the catalytic domain, inhibit cellular PKC activity, and, thus, interfere with the PKC-overactivation-driven pathological processes.

Curcumin inhibition of PKC may impose several therapeutic implications in diabetes, particularly in case of diabetic retinopathy. Increased PKC activation has been demonstrated in experimental diabetic retinopathy studies (Kowluru 2001) and several PKC inhibitors (PKC*b*II) are being tested for their efficacy and safety in clinical trials (Frank 2002). Specifically, in diabetic retinopathy, both the genesis and the advanced stage of proliferative diabetic retinopathy (PDR) have been hypothesized to be a result of increased oxidative species (Armstrong *et al* 1998) or to be associated with ischemia-perfusion injury at the boundaries of perfused and nonperfused retina, which leads to both increased oxidative species and neovascularization. Since curcumin is also an angiogenesis inhibitor and inhibits multistep progression of angiogenesis *in vitro* and *in vivo*, one cannot underestimate its therapeutic potentials. It appears that the pleiotropic effects of curcumin are at least partly due to inhibition of the transcription factors NF-kappa B and AP-1 (Bierhaus *et al* 1997). Okamoto *et al* (2002) reported that curcumin prevents the AGE-induced increase in NF-*k*B and AP-1 activity, vascular endothelial growth-factor (VEGF) mRNA up-regulation and the resultant increase in DNA synthesis in microvascular endothelial cells. Any drug with multiple actions, such as antioxidant and anti-angiogenic effects, is an ideal formulation in the treatment of diabetic retinopathy.

Our results demonstrate that antioxidant effects of curcumin involve, among other mechanisms, the inhibition of Ca^{2+} influx and PKC. This information extends the known cellular actions of curcumin to potentially multiple therapeutic targets. Pharmacologically, curcumin is reported as safe (Aggarwal *et al* 2003). A phase-one human trial with 25 subjects using up to 8000 mg of curcumin per day for 3 months found no toxicity from curcumin (Cheng *et al* 2001). Five other human trials using 1125–2500 mg of curcumin per day also showed no toxicity (Chainani-Wu 2003). Xu *et al* (2003) reported that curcumin inhibited the proliferation of activated hepatic stellate cells and this thas been mediated by curcumin induction of the gene expression of PPAR*g* and PPAR*g* activation. The PPAR*g* agonists, thioazolidenediones (which are primarily antidiabetic agents) are claimed to offer multiple therapeutic benefits via their beneficial vascular and anti-inflammatory effects (Martens *et al* 2002). In this context, curcumin on further scientific validation appears to have multiple potential in the treatment regimen of diabetes and its complications, which constitutes a collection of metabolic, vascular and inflammatory defects.

Acknowledgements

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